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## ERRATUM

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Line 13 page 283 the word *hagfish* should be corrected to *axolotl*

Correct line is printed below and should be cut out and pasted over the incorrect line

5% reported herein In the axolotl they

# The Changing Pattern of Basophilia in the Mouse Uterus from Mating through Implantation

LOIS JEAN SMITH[1]
*Department of Anatomy, Albert Einstein College of Medicine, New York, New York*

*ABSTRACT* Changes in RNA localization and concentration in mouse uteri taken from one-half to seven and one-quarter days after mating were visualized as changes in basophilia following staining by a histochemical method specific for RNA. The basophilia of the luminal epithelium falls abruptly between one half and two days and is still lower at two and three-quarter days. By 2 and three-quarter days, however, that of the stroma and especially that of the subepithelial endothelial cells is increasing. A further slight increase is found at three and three-quarter days and by four days a band of subepithelial stromal cells, wider in the antimesometrial than in the mesometrial portion of the uterus, is highly basophilic. At four and one-quarter days, when a distinct decidual reaction is evident, cells in the primary decidual zone are less basophilic than at four days. During the remaining period of observation cells in different regions of the uterus show increased and often later decreased basophilia. It was concluded that these results are in complete accord with those from a recent biochemical study of changes in RNA in the intact rat uterus during the interval from induction of pseudopregnancy through deciduoma formation if the difference in timing in the two species is taken into account.

Although Jeener reported in 1948 that estrogen administration leads to increased RNA synthesis in the rodent uterus, it was not until the function of the various RNAs in the chain from DNA to specific protein was uncovered that the possible significance of this finding was fully appreciated. Numerous recent data from many laboratories now strongly support the view that the cyclic differentiation of the adult uterus may be based on cyclic activation of specific genes by estrogen. These findings include information on the time sequence of new syntheses of RNA, DNA and protein after estrogen administration, on the effects of concurrent administration of actinomycin D and puromycin on RNA and protein synthesis, and on the demonstration that estrogen administration results in differential protein synthesis in a variety of tissues.

As a consequence of these studies the role of estrogen during pregnancy and of other factors involved in uterine differentiation are being investigated with renewed interest. Most of the newer data on the sequence of syntheses of RNA, DNA and protein in the interval from mating to decidua formation have been obtained from biochemical analyses of whole rodent uteri, however, and there appears to be a paucity of information on localized changes in concentration during this period of differentiation. This is particularly so with respect to RNA since data based on the affinity of nucleoproteins for basic dyes under controlled staining conditions seem to consist of a report on three rats of unstated stages of pregnancy (Wislocki and Dempsey, '45) and a study devoted principally to the trophoblast giant cells in which methylene blue of unspecified pH was used (Alden, '48). There is also an account of the differentiation of the metrial gland in which passing reference is made to the stainability of the endometrium (Baker, '48) and a description of basophilia in the uterus at a stage just prior to the time of attachment of the allantois (Bulmer and Dickson, '61).

In the present paper are to be found the results of a histochemical study of RNA in the endometrium of the pregnant mouse from one half to seven and one quarter days after mating. For the study the method of Flax and Himes ('52) involving the use of the metachromatic dye Azure B

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was chosen Their results indicate that this method distinguishes between RNA DNA and acid mucopolysaccharides and is semi quantitative with respect to RNA During the course of the study, basophilic cells which seem to be precursors of metrial gland cells were found at younger stages than previously reported A full discussion of these observations and those of a parallel study of glycogen distribution are to be found in Smith ( 66)

## MATERIALS AND METHODS

F1 females from several different types of crosses were exposed to males from the same crosses between 5 P M and 10 A M to obtain uteri four and one half to seven and one quarter days post coitum and between 9 P M and 2 A M to get earlier stages Mating was arbitrarily assumed to have occurred at 1 A M of the day a vaginal plug was found and the females were sacrificed from one half to seven and one quarter days later Uteri from two pregnant females only were examined at one half and two days for each of the other times at least five were available All females had had one or two previous litters before being used in this study

Uteri to be stained for basophilia were fixed in Carnoy s fluid sectioned serially either transversely or longitudinally and stained by the Azure B method of Flax and Himes ( 52) Ribonuclease digestion was performed at some stages Examples stained with Delafield s and iron hematoxylin and by the Feulgen method were available for many of the stages up to seven and one quarter days and for eight and one half and nine day stages as well

## RESULTS

The following account of uterine differentiation during early pregnancy includes what appear to be significant stages in trophoblast development with respect to uterine trophoblast interactions For concurrent stages in embryo development see Flax ( 53) and Smith ( 56) for the interval from mating through the third day of pregnancy and Snell ( 41) for the remaining period investigated These embryonic stages are also illustrated diagrammatically in figures 1–10

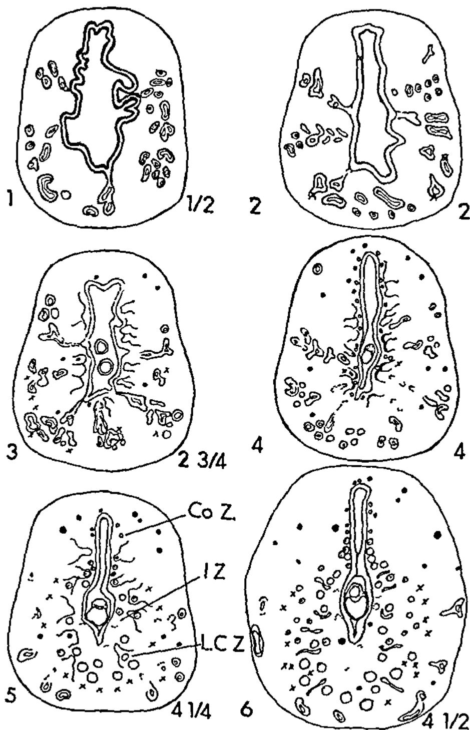
### *One half day after mating*

At 12 hours after mating almost no glands empty into the mesometrial one quarter to one fifth of the uterine lumen and because of this the endometrium appears to be divided more or less distinctly into mesometrial and antimesometrial portions (fig 1) In addition in the mesometrial region and in particular around the lumen the stromal cells are more tightly packed than they are elsewhere in the mucosa and tend to have rounder nuclei and more cytoplasm than do the antimesometrial cells The basal layer contains no glands In it the spindle shaped cells have oval nuclei and are widely spaced

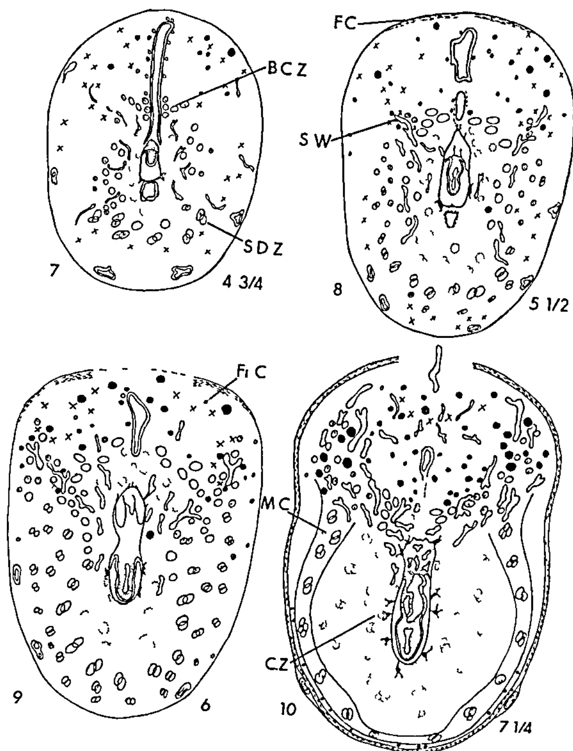
Neutrophilic polymorphonuclear leucocytes, lymphocytes, macrophages and reticular cells are present throughout the endometrium The neutrophils appear to be almost as plentiful as the stromal cells and like them decrease in concentration from lumen to periphery The lymphocytes are found mainly in the lymph spaces at the periphery The cytoplasm of these cells of the macrophages which contain a light sprinkling of yellow granules and of the stromal cells show very little affinity for Azure B while cells which seem to be reticular cells or immature plasma cells stain intensely The latter are found in highest concentration around the glands

The high columnar cells of the luminal epithelium show greater basophilia of both apical and basal cytoplasm at this time than at any other stage studied and probably greater basophilia than is shown by any other uterine cell examined The basophilic material which is removed by ribonuclease also stains much more metachromatically than does the approximately equally heavily stained material of the em

Figs 1–6 Diagrammatic cross sections of pregnant uteri from one half to four and one half days after mating The mesometrial region is uppermost Luminal and glandular epithelium are indicated by double lines capillaries by single lines Mitotic figures are symbolized by (×) dark and metrial gland cells by (●) compact zone cells by (○) other mononuclear cells increasing in size and with 2 to 3 basophilic nucleoli by (◉) Further increase in cell size is shown by the increased size of the symbols Degeneration or loss of basophilia is indicated by broken out lines Zones indicated on the drawings on the day they first appear are: compact zone *Co Z* implantation zone *I Z* large cell zone *L C Z*




Figures 1–6




Figs 7–10 Diagrammatic cross sections of pregnant uteri from four and three fourths to seven and one fourth days after mating At two-thirds the magnification of figures 1–6 Additional cell types seen during this period are binuclear cells ( ) and multinuclear cells ( ) Additional zones found are basophilic cell zone *B C Z* central zone *C Z* fibrous capsule *F C* fibrinoid capsule *Fi C* multinuclear cell zone *M C* sinusoid wings *S W* secondary decidual zone *S D Z*

bryonic cells of later stages Hall (60) noted similar differences in metachromasy in stromal and epithelial cells after staining with toluidine blue The cytoplasm of the glandular epithelium is also highly basophilic and more so basally than apically No neutrophils are found in the glandular epithelium but there are large numbers often in patches between the luminal epithelial cells

*Two days*

The principal change to be seen in the non-epithelial cells of the mucosa at this age is a marked decrease in the number of neutrophils those present often have a pycnotic nucleus

The luminal epithelium however is very much changed (fig 2) The massive folding found at 12 hours has disappeared the height of individual cells is less and cytoplasmic basophilia has dropped sharply especially in the basal portion of the cells In places the orderly arrangement of the centrally located nuclei is disrupted and the epithelial surface generally shows a rough and irregular outline In addition large basally located vacuoles containing pycnotic nuclei are present between apparently normal epithelial cells There are occasional mitotic figures in the luminal epithelium and many in the distal portion of the glandular epithelium

*Two and three quarter days*

Of the six uteri taken at this interval after mating the four in which the morulae are most dispersed show the most intense basophilia

In all these uteri the size and basophilia of the cells of the capillaries adjacent to the uterine lumen are markedly greater than at two days the capillaries tending to stand out against a background of palely staining stromal cells (fig 3) The latter also seem to show slightly increased amounts of cytoplasm and increased basophilia Peripherally the diameter but not the basophilia of the capillaries has increased Throughout the endothelium mitotic figures are common and they are also frequent around the glands In the latter location however it is impossible to determine what cell types are involved

The number of polymorphs now present in the stroma has fallen sharply but lymphocytes flood some areas around large capillaries In addition around the glands there are both mature plasma cells and a new type of basophilic cell usually distinct from the plasma cell on the basis of nuclear stainability and cell shape This new type of cell described more fully in Smith (66) varies continuously in size from that of a large lymphocyte with a narrow rim of basophilic cytoplasm to a cell with a wide band of distinctively striated cytoplasm Cells with such striated cytoplasm seem to be small metrial gland cells and will be so designated henceforth to distinguish them from the smaller "dark" cells of the new type

The luminal epithelium at two and three quarter days is more highly columnar than at 48 hours and the vacuoles present at the earlier stages are rare In addition increased numbers of mitotic figures are present in the epithelium Basophilia however is even more reduced Finally the epithelium of the neck of some of the glands is now disorganized and shows very reduced cytoplasmic basophilia Such glands often appear occluded adjacent to the uterine lumen Distally however the lumens of all glands are enlarged and basophilia is confined to the basal cytoplasm Mitotic figures are fairly frequent distally

*Three and one half to four days*

Both the basophilia and the size of the cells in three and one half day old pregnant uteri are very similar to those in two and three-quarter day old uteri In uteri taken between three and one half and three and three-quarter days post coitum however and containing blastocysts whose blastocoel is slightly larger than the inner cell mass the luminal epithelial cells next to the blastocysts are lower and show less basophilia than those only a short distance away and a few contain shrunken nuclei This portion of the epithelium can often be seen to be connected to the plump basophilic trophoblast cells of the blastocysts by fine protoplasmic strands Parenthetically in sections fixed for staining by the PAS technique the zona pellucida around blastocysts of this size appears cracked

The zona is apparently lost very soon after this for in uteri taken between three and three quarter and four days after mating and containing embryos with a somewhat larger blastocoel it is clear that the em bryos are firmly fixed to the epithelium by three or four enlarged and vacuolated trophoblast cells At this stage of nidation the uterine epithelial and stromal cells are separated in the region immediately around the embryos Interestingly, there also seems to be a minor mobilization of lymphocytes beneath the epithelium Some of the nu clei of these lymphocytes are irregular lobulated or fragmented

*Four days*

In uteri taken at four days the blasto cysts are pressed tightly against the epi thelium into which blunt processes from the trophoblast extend In the region of such blastocysts the organization of the epithelium as a single layer of cells has clearly been lost and some of the epithelial nuclei are pycnotic In addition there are a few spherical bodies which stain heavily for DNA with both Azure B and the Feul gen reagent in the basal portion of the epi thelium while under the epithelium there are large numbers of cells with irregularly shaped nuclei and similar pycnotic bodies It is assumed that both are the remains of the invading lymphocytes

At this stage a subepithelial strip slightly wider in the vicinity of the implant ing embryo than elsewhere is sharply de limited from the remainder of the stroma by its greater Azure B stainability (fig 4) The stromal cells in this strip particularly those in the antimesometrial area are clearly larger than those in earlier stages are more tightly packed and are now as basophilic as the cells lining the capillaries The latter cells have also enlarged and so much so that some of the capillaries adja cent to the epithelium lateral and anti mesometrial to the embryos appear oc cluded There are also a few mitotic figures in the stroma at the level of the embryo

*Four and one quarter days*

Both the inner cell mass and the blasto-coel of embryos of this age have enlarged The blastocoel is often collapsed however and the much flattened trophoblast cells surrounding such a blastocoel are sepa rated from the epithelium Basophilic proc esses from these trophoblast cells can nevertheless be seen deep within the less basophilic epithelium Such processes fre quently contain elongate, pycnotic nuclei and similar nuclei lie between the blasto-cyst and the epithelium

At this stage the size of the cells in a wide strip of mucosa lateral and antimeso-metrial to even the least developmentally advanced of the embryos is clearly greater than that of the cells in the same region at four days Furthermore this strip can be divided, roughly into a subepithelial and a vascular layer since the capillaries im mediately beneath the luminal epithelium no longer seem patent while those located more distally are open and are larger in diameter than any found elsewhere in the endometrium (fig 5) The subepithelial layer seems to correspond to the primary decidual zone of Krehbiel (37) and in more advanced four and one quarter day uteri the stromal cells of this region are less basophilic than are those in the vascu lar layer This loss of basophilia seems to be the first sign of the formation of the implantation zone (Krehbiel 37)

Immediately external to the vascular layer where the cells are less tightly packed there are large numbers of mitotic figures These figures are especially fre quent in a cup-shaped area antimeso-metrial to the embryo and in the least ad vanced of the four and one quarter day uteri the largest cells of the uterus are found here and in the vascular layer just mesometrial to it These cells are uni nuclear and have 2 to 3 very large nucleoli In older four and one quarter day uteri all of the cells of the vascular layer are like this and are clearly larger than those else where in the uterus The vascular layer will hereafter be called the large cell zone

Immediately mesometrial to the embryo the capillaries are still open adjacent to the luminal epithelium so that in this re gion there is no primary decidual zone only a large cell zone Still more mesometrially around the mesometrial portion of the lu men, the subepithelial cells decrease in size rather abruptly and are more closely packed than are cells in other regions This

region will henceforth be called the com pact zone

By this stage also the luminal epithe lium can be divided into the four regions mesometrial transitional implantational and antimesometrial described by Krehbiel (37) at the start of the sixth day in the rat Basophilia is lowest in the implanta tional epithelium which is detached from the underlying stroma Finally in the re gion of implantation normal appearing glandular epithelium is present only in the basal layer closer to the lumen nothing but pale cords remain

*Four and one half days*

By this time as the result of the hyper plasia and hypertrophy of the stromal cells immediately mesometrial to the embryo the epithelial sac surrounding the embryonic crypt is almost completely cut off from the main portion of the lumen (fig 6) Furthermore the organization of the epi thelium forming the sac has broken down and the cells are either jumbled together or missing When missing their place is taken by the enlarging vacuolated trophoblast cells which now encircle the blastocoel The latter often contain fragmented epithelial cells Finally the width of the zone of cells showing reduced basophilia (the implantation zone) has increased con siderably and now completely surrounds the embryo

*Four and three quarter days*

Four trends which presage the future direction of the decidual reaction and the formation of the definitive placenta are present at this time (fig 7) First some of the cells in the cup-shaped area of the large cell zone antimesometrial to the em bryo are now binuclear Krehbiel (37) called the zone in which the binuclear cells appeared the secondary decidual zone Sec ond although mitotic figures are still fre quent at the periphery of the mucosa lat eral to the embryo for the first time a very large number is present mesometrial to the embryo Some are in endothelial cells in the region of sinusoid development (see below) and others may be in stromal cells around the enlarging capillaries Still others are in the compact zone but whether in endothelial or stromal cells could not be determined Third a polarization of en larging capillaries suggests the beginning stages of the sinusoid wings described by Krehbiel (37) Fourth since the enlarged capillaries are not found within the portion of the large cell zone mesometrial to the embryo and adjacent to the transitional epi thelium this area as a whole appears to be the most basophilic in the uterus This portion of the large cell zone will be called the basophilic cell zone henceforth

At this time the dissolution of the lumi nal epithelium has progressed to the point where the trophoblast is in direct contact with the stroma everywhere except meso metrially in the region of the inner cell mass and antimesometrially where the embryo still rests on a shelf of compressed epithelium In addition the trophoblast cells surrounding the blastocoel are be coming transformed into giant cells and a few processes from them have penetrated between the decidual cells of the implanta tion zone

*Five and one half days*

Thin cords of cells projecting from the ectoplacental cone have by now grown up the narrow channel still connecting the embryonic crypt with the uterine lumen al most as far mesometrially as the basophilic cell zone (fig 8) The cells at the periphery of the trophoblast cords also seem to have eroded some of the sinusoids formed from the enlarged capillaries seen at four and three-quarter days because maternal red blood cells are now present between the trophoblast cords Large trophoblast cells are easily distinguishable from the large decidual cells by virtue of their greater basophilia and large extremely lobulate nucleoli See Alden (48) also

At this time after staining by the PAS technique the basophilic cell zone can be seen to correspond to the glycogenic area of Krehbiel (37)

*Six days*

The degree of basophilia and the cell types characterizing each region of the pre placental pregnant uterus are sharply dis tinct at this time (fig 9) External to the sinusoid wings and mesometrial to the compact zone some of the fibroblasts of the basal layer now appear compressed and

highly basophilic These compressed cells are apparently what Bridgman (48a) described as a fibrous layer The compact zone which will form the mid portion of the decidua basalis contains two types of stromal cells small tightly packed mononuclear cells which seem to differ from those seen at 12 hours only by a slight increase in size and a great increase in basophilia and many medium sized metrial gland cells The latter stand out from the fusiform stromal cells by virtue of their shape vacuolization kidney shaped nucleus and large nucleoli This zone now also contains many mitotic figures

There is a sharp discontinuity in size between the cells of the compact zone and of the basophilic cell zone immediately antimesometrial to it The basophilic cell zone is made up of cells of giant size high basophilia and a single nucleus with 2 or 3 very large, compact and basophilic nucleoli The area of the sinusoid wings contains smaller cells similar to those in the basophilic cell zone with respect to their basophilia and nuclear morphology and some metrial gland cells

In the widening implantation zone the poorly basophilic cells have shrunken nuclei and pale nucleoli Processes from trophoblast cells seem to be lying within the eroded capillaries of this region External to the implantation zone and lateral and antimesometrial to the embryo the cells are moderately basophilic and usually binuclear They are also easily distinguished by their multiple small pale nucleoli

*Seven and one quarter days*

The compact zone has increased in size conspicuously if the remnant of lumen still remaining at this time is used as a marker (fig 10) Here the sinusoids have become much more plentiful although they remain relatively small as compared to those in the sinusoid wings and stromal cell number is greatly increased The number of dark and medium sized metrial gland cells now present is striking Even larger metrial gland cells and very many dark cells are present throughout the region of the sinusoid wings The latter contain large numbers of leucocytes

A few of the now giant cells of the basophilic cell zone and of the smaller cells in the sinusoid wings are binuclear at this stage the nucleus of others being irregular in shape All have remained highly basophilic however and still have only 2 to 3 large basophilic nucleoli A small number of metrial gland cells is now seen in this zone

Almost all of the cells of the endometrium lateral and antimesometrial to the embryo except those immediately beneath the basal layer are also greatly enlarged at this stage and most contain multiple small pale nucleoli The largest of these maternal giant cells are located at the periphery of this area and some are tri or tetranuclear while many have irregular or lobulate nuclei Basophilia is highest here also The small cells next to the basal layer are mononuclear and contain 2 to 3 nucleoli These small cells have vacuolated cytoplasm as do the small mononuclear cells immediately external to the sinusoid wings and beneath the fibrous layer These cells probably make up the fibrinoid capsule of Bulmer and Dickson (61)

At this time the cells forming the mesometrial roof of the ectoplacental cavity (the cytotrophoblast or the lamina of Everett 35) are highly basophilic as are the peripherally located giant cells Both are in sharp contrast to the pale vacuolated spongiotrophoblast cells internal to these regions and to the adjacent maternal cells which the giant cells often appear to be surrounding The beginning of central zone formation (Everett 35) is thus evident

*Eight and one half days*

The allantois has made contact with the lamina of the ectoplacental cone by now and the central zone is several cells wide Polymorphs and metrial gland cells are present in the maternal blood spaces of the mesometrial portion of the zone

*Nine and one half days*

By nine and one half days after mating the ectoplacental cone extends in the form of a wedge up the original uterine lumen almost to the level of the compact zone and most of the cells in the basophilic cell zone have either been pushed laterally or phagocytized The giant cells of the

*trophoblast* abut therefore mesometrially on the compact zone with its many large metrial gland cells and laterally on the sinusoid area which also contains very large metrial gland cells Some of the latter appear to be free in the maternal sinuses Enlarged endothelial cells encircled by a cuff of metrial gland cells such as was de scribed by Dickson and Bulmer (61) and Bridgman (48b) are present in the com pact zone at this time

### *Summary of the changing pattern of basophilia*

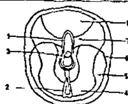
Mature plasma cells are present around the uterine glands in fairly high numbers by two and three-quarter days after mating and some are still found at seven and one quarter days Since it seems reasonable to assume that the basophilia of these cells remains rather constant during this period the intensity of their staining was used as a standard of comparison for estimating relative changes in the basophilia of other uterine cells (table 1) It must be stressed however that the values in the table are arbitrary and are intended to show only the direction of the change i e either in creases or decreases in basophilia It should also be noted that the values in the table are for the cytoplasmic basophilia of individual cells within a particular area

TABLE 1

*A summary and rough estimate of the changes in basophilia of individual cells within the pregnant uterus from one half to seven and one-quarter days after mating The basophila of the mature plasma cell was taken as a constant and given the value 4 less basophilic cells received lower numbers Areas within the uterus have been identified both by number on the drawing and in terms of their prospec tive fate Where values are shown as 1-0 the first figure is for more medially located cells*

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 |
|---|---|---|---|---|---|---|---|---|---|
| D ys f m *mating* | Luminal epi thel um | Gl ndul epi thelium | Prim d zone | Large cell zone | Multi nucl cell zone | B al zone | B so- philic c ll zone | Compact z e | T oph |
| ½ | 7 | 3 | 2 | 2 | 1 | 0 | 2 | 2 | |
| 2 | 4* | 4 | 2 | 2 | 1 | 0 | 2 | 2 | |
| 2¾ | 2 | 2–4 | +3<br>2 | +3<br>2 | +3<br>1 | 0 | +3<br>2 | +3<br>2 | |
| 3½ | x2<br>2½ | 0–4 | +4<br>3 | +4<br>2½ | +3<br>1½ | 0 | +4<br>3–2 | +4<br>3–2 | 4 |
| 4 | x2<br>2½ | 0–4 | +4<br>4 | +4<br>3 | 2 | 0 | +4<br>4–2 | +4<br>4–2 | 4 or 2 |
| 4¼ | x1<br>2 | 0–4 | 4 or 2 | 4 | 2 | 1 | 4–2 | 4–2 | 4 or 2 |
| 4½ | x0<br>2 | 0–4 | 2 | 4 | 2½ | 1½ | 4–2 | 4–2 | 5 |
| 4¾ | x0<br>2 | 3 | 1 | 3–4 | 3 | 2 | 5–2 | 4–2 | 5 |
| 5 | x†<br>2 | 3 | 0 | 2–4 | 3½ | 2 | 5–2 | 4–2 | 5 |
| 5½ | 2 | 2½ | 0 | 1–4 | 4 | 2½ | 4–5–3 | 4–2 | 5 |
| 6 | 2 | † | † | 3 | 4½ | 3 | 3–6–4 | 5 | 6 |
| 7¼ | 1 | † | † | †–3 | 5–6 | xx7 | 6 | 5 | 6 |

† mi ing
api al, in deg nerating cells
norm l cell
\+ capill ri s only
x near embry
xx fibroid c p ule only



one allows for the difference in the rate of development in the two species The marked similarity in the decidual morphology of the two species would appear to make this a valid comparison in spite of some evidence that hormonal differences exist between them Furthermore Shelesnyak and co-workers (Marcus et al 64) found that the histamine content of the early pregnant uterus was different from that of pseudopregnant uteri at comparable times and concluded that the hormonal influences on the two types of uteri were also different Atkinson and Hooker (45) however came to the opposite conclusion in their study of the mouse

In the present histochemical study the basophilia of the epithelium particularly of the luminal epithelium was found to fall abruptly between 12 and 48 hours after mating (although the low point had probably not been reached by 48 hours) while that of the remaining cells of the endometrium remained fairly constant The drop in basophilia in the luminal epithelium was so great that it seems likely that a decided drop in RNA content might well be found to have occurred between 12 and 48 hours if biochemical analyses of entire uteri were done at these two times An additional reduction would probably come about as a consequence of the morphological changes during this interval since they were similar to those seen between estrus and late metestrus (Allen 22) and Drasher (52) reported a sharp drop in RNA P/DNA P ratios between estrus and diestrus in the mouse Except for a difference in timing therefore these results agree with those of Shelesnyak and Tic who found a drop in RNA content of entire uteri immediately after inducing pseudopregnancy at proestrus with the lowest value being reached at what would be the time of diestrus in the normal cycle Moreover their data for this period seem identical to those they obtained at comparable times in the normal cycle

The two sets of data on RNA content in uteri undergoing "sensitization" to the stimulus for decidualization also seem to agree except that this process appears to begin earlier in the mouse than in the rat In the histochemical study the intensity of endometrial basophilia (excluding that of the epithelium) was found to be visibly greater at two and three-quarter days than at two days and it rose slowly until the fourth day The intensity of basophilia in the luminal epithelium on the other hand was still lower at two and three quarter days than at two days after mating but it had risen somewhat by three and one half days although in the region of the embryo it continued to rise only until about three and three-quarter days As the detailed description of the situation during these hours indicated this pattern of increase in intensity of basophilia is quite different from that seen in a normal cycle That is the increase is not one of increasing basophilia in multiplying luminal and glandular epithelium but is primarily one of an intensifying basophilia in endothelial cells which are also increasing in size and number and secondly one of an intensifying basophilia in stromal cells which are increasing in size and number too but more slowly In addition the basophilic metrial gland cells are beginning to differentiate at two and three-quarter days It is these changes then together with an increase in capillary size which is also found at two and three-quarter days which results in a uterus competent to respond "sensitized" to the stimulus for decidualization

With respect to the level of RNA in the rat uterus during the period of sensitization from the data of Shelesnyak and Tic RNA concentration seems to remain very low between Day 1 and Day 3 or until approximately three and one half days after the induction of pseudopregnancy by the timing used in this mouse study After that there appears to be a slow rise which seems to correspond to that seen between two and three-quarter and four days in the mouse Before the decidualization stimulus was given at four and one half days there had been an "elevation of the content and concentration of protein RNA and DNA over the basal level of dioestrus"

Since it is currently proposed that the buildup of RNA is mediated by estrogen (Ui and Mueller 63 and Frieden 64 for additional references) the hormonal state during the period from mating or from the time pseudopregnancy is induced to the time the stimulus for decidualization is given is of course of interest Shelesnyak

and are not for the area as a whole The scores therefore, reflect only imperfectly the major changes in the amount of RNA which occur in the pregnant uterus during the period covered by this study

## DISCUSSION

### *General uterine organization*

From the results reported here, it is apparent that the endometrium even at 12 hours after a successful mating is not homogeneous Even at this time the mesometrial portion differs from the antimesometrial in the relationship of glands to lumen and to stromal cells and probably also in the state of the stromal cells around the lumen In addition both Williams ( 48) and Young ( 51 ) found differences in vascularization in the two regions in the rat

Examination of successively older stages reveals that it is the fusiform stromal cells together with the endothelial cells adjacent to the degenerating gland necks which early become basophilic and enlarged when the uterus is becoming sensitized to decidualization and that it is these same stromal cells in which the decidual reaction is first localized following the stimulation of trophoblast attachment In other words only the stromal cells in the region containing glands seem to differentiate into maternal giant cells These large cells are also the shortest lived of the decidual cells and many have disappeared by the time placental circulation begins

The stromal cells around the mesometrial lumen on the other hand never get very large and show few morphological signs of differentiation at least in the interval studied Unlike the large antimesometrial multinucleolate stromal cells however it seems reasonable to suppose they provide an environment suitable for the differentiation of the precursors of the metrial gland cells which are so plentiful in this area in later stages of uterine development It should be noted here that a possible origin of metrial gland cells from cells other than *in situ* mesometrial stromal cells is suggested in Smith ( 66)

### *Cytology of the decidual cells*

In Krehbiel s ( 37) now classic study of the pregnant rat uterus it was reported that glycogen bearing cells are found almost exclusively in the mesometrial portion of the decidua while cells containing large amounts of lipid are characteristic of the antimesometrial decidua The present work discloses what appears to be a further and functionally significant difference between the two areas in the mouse This is that the multinuclear antimesometrial cells which possess 2 to 3 highly basophilic nucleoli per nucleus in early stages of differentiation in later stages contain many small pale nucleoli and as cell size increases in this area basophilia tends to decrease The mesometrial decidual cells of the basophilic cell zone and sinusoid wings on the other hand are characterized by the presence of only 2 to 3 large basophilic nucleoli per nucleus all during differentiation and cytoplasmic basophilia tends to remain constant as cell size increases Vacuoles however are evident in the cytoplasm when glycogen makes its appearance in these cells at five and one half days

The recent electron microscopic study of Jollie and Bencosme ( 65) in which the fate of the glycogen containing cells of the primary decidual zone in the rat was followed appears to offer no contradictions to these findings although it is difficult to determine corresponding cell types and timing in the two studies Subepithelial cells in the primary decidual zone in the mouse however lose their basophilia as soon as they begin to enlarge then become glycogen laden and finally are phagocytized by five and one half days after mating (Smith 66)

### *Pattern of basophilia in the uterus*

The changes in basophilia observed in the endometrium of the mouse between 12 hours and seven and one quarter days after mating are on the whole what would be expected on the basis of the association between the basophilia seen after Azure B staining and RNA (Flax and Himes 52) and the present ideas on the relationship of RNA to protein synthesis during differentiation and growth

The changes also parallel in striking fashion the changes in RNA content during artificial decidualization of the progestational rat uterus as determined biochemically by Shelesnyak and Tic ( 63) if

subepithelial stromal cells with respect to size is evident As the data indicate from four and one quarter days on the increase in cell size and number and in differentiation continues unchecked in the implantation site as some cells gain basophilia while others are losing it

It seems necessary to emphasize that an interaction between the embryo and the uterus is most probably the stimulus for decidualization in view of the hypothesis of Shelesnyak (Marcus et al 64) that it is the estrogen surge which releases histamine from mast cells which is the stimulus rather than the trauma of implantation or an exogenous trauma in the case of deciduoma induction (See Noyes et al 63 for a discussion of the active role of the rat egg *in implantation* ) Nevertheless in Shelesnyak and Tic s study an exogenous trauma was given at four and one half days and following this there was a second and rapid rise in RNA concentration and deciduoma formation As was previously *indicated it was impossible to so accurately* pinpoint the time of onset of the sharp rise in basophilia seen in this mouse study

With respect to a later stage an apparent difference in the pattern of basophilia between the rat and the mouse must be mentioned Bulmer and Dickson ( 61) report greater basophilia just outside the implantation zone of the antimesometrial decidua of the *nine day rat than more peripherally* This is the reverse of the finding in the mouse at a similar stage i e at seven and one-quarter days Their material however was preserved in formalin containing fixatives while the mouse material was not It is also possible that their description of this stage was taken from material stained with a stain other than Azure B

ACKNOWLEDGMENTS

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has proposed that a brief estrogen surge during the period of progesterone domination and different from that required for priming the uterus for proliferation is responsible for the transient sensitization of the rat uterus to decidual induction (Shelesnyak et al , 63) Furthermore they suggest that their data indicate that the surge occurs in their strain at about 12 noon of Day 3 (or at three and one half days by my timing) Zeilmaker ( 63) who ovariectomized pregnant rats obtained similar results This is about the time of the beginning of the transient elevation in RNA seen in Shelesnyak and Tic s study In addition Mayer ( 63) was able to induce nidation at will within 48 hours after the injection of estradiol into pregnant rats ovariectomized on Day 3 (same timing as Shelesnyak) and maintained on progesterone Finally Yochim and DeFeo ( 63) have shown that exceeding the optimal level of estrogen results in a failure of deciduoma formation in rats

Unfortunately no similar data are available for the mouse that will allow as clear cut a correlation to be made between the time of the estrogen surge and the time basophilia begins to increase in the endothelial cells (at approximately two and three quarter days in the mice used in this study) That estrogen is necessary for implantation in the mouse was shown by Bloch ( 58) and Smithberg and Runner ( 60) Furthermore the latter authors were also able to induce nidation with progesterone alone if the ovaries were removed one or two days post coitum (Smithberg and Runner 56) which suggested to them that estrogen is needed at least 1 or 2 days earlier in the mouse than it is in the rat (Smithberg and Runner 60) This agrees with the morphological data of Atkinson and Hooker ( 45) who concluded that estrogen is at its highest at the time of mating falls to a low level on the next day and has almost disappeared by the third day of pregnancy

Until more exact endocrinological data are available for the mouse therefore the time of entrance of the morulae into the uterus may be the best indication that the same stages of embryo and uterine differentiation have been reached in the two rodents Morulae had entered the mouse uterus slightly before two and three quarter days post coitum and an increase in endothelial basophilia was evident at two and three quarter days According to most investigators morulae enter the rat uterus around three and one half days after mating and Shelesnyak and Tic s data suggest RNA concentration begins to increase by this time

The corresponding times at which the stimuli for decidualization were given the mouse and the rat resulting in a second and very sharp rise in basophilia in the mouse and in RNA in the rat are even more difficult to determine This is partly because it is impossible to be sure on purely morphological grounds what constituted the stimulus in the present study The sequence of changes in the blastocyst in the epithelium next to the blastocyst and in the subepithelial stromal cells between three and one half and four days after mating however suggests that the stimulus for decidualization in the present study was received slightly before the fourth day These changes also suggest that the stimulus was the contact between a blastocyst differentiated to the extent of being able to adhere to the epithelium and the epithelium overlying a competent stroma With respect to this interpretation of what constitutes the stimulus for decidualization it is most important to stress that localized changes in the epithelium in the underlying basement membrane and in the lymphocytes are evident before the subepithelial stromal cells attain maximum basophilia These cells have reached this state by the time blunt processes from the trophoblast can be seen within the epithelium (at 4 days) and at this time stromal cell volume and number are very obviously increasing Unless this increase in stromal cell volume and number constitutes sensitization of the uterus to decidualization rather than the beginning stages of decidualization therefore the shallow penetration of the epithelium by the trophoblast cells at four days and the presence of the invasive cells (Wilson 63) at four and one quarter days seem to follow the stimulus It must be pointed out however that it is not until four and one quarter days after mating that a clear difference between mesometrial and antimesometrial

subepithelial stromal cells with respect to size is evident As the data indicate from four and one quarter days on the increase in cell size and number and in differentia tion continues unchecked *in the implanta tion site as some cells gain* basophilia *while others are losing it*

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# Metrial Gland and Other Glycogen Containing Cells in the Mouse Uterus Following Mating and Through Implantation of the Embryo

LOIS JEAN SMITH

*Department of Anatomy Albert Einstein College of Medicine New York New York*

*ABSTRACT* Mouse uteri and embryos from 12 hours to seven days after mating were examined for RNA and glycogen following specific histochemical staining Because of their greater basophilia calls present throughout the endometrium which seem to be metrial gland cell precursors could be distinguished as early as two and three-quarter days At this time the uterus is just beginning to undergo "sensitization to decidualization By seven days these precursor cells and metrial gland cells proper have disappeared from all regions of the endometrium except that of the future decidua basalis Glycogen is present in cells which may be the metrial gland cell precursors plasma cells and polymorphonuclear leucocytes as well as in the lumens and cells of the uterine glands at three and one half days Between four and one-fourth and five and one-half days glycogen is found in the primary decidual zone By six days there is a heavy deposit in the "glycogenic areas" At seven days many of the metrial gland cells in the future decidua basalis are PAS-positive These findings are discussed and the possible origin of the metrial gland cells from a cell of the lymphocytic series is suggested

Differences in the basophilia of several cell types in the uterus made possible the discovery that cells which seem to be metrial gland cell precursors are present in small numbers throughout the endometrium of the mouse at 65 hours after mating (Smith 66) At this time the conceptuses have just entered the uterus which itself is just beginning to transform into an organ into which the embryos can implant Metrial gland cells and their precursors have not previously been reported anywhere in the mouse or rat uterus at such an early stage of pregnancy At later stages these cells are concentrated around blood vessels in the decidua basalis and in the metrial gland proper

Mature metrial gland cells are characterized by a distinctively striated cytoplasm and contain both glycogen and PAS-positive diastase resistant granules The function of these cells and whether or not the metrial gland is in fact a gland is still being debated Investigators from Jenkinson ( 02) through Padykula and Richardson ( 63) have discussed the possible contribution of the intracellular glycogen to the nourishment of the embryo but have been unable to agree on any one hypothesis Wislocki et al ( 57) have suggested that the diastase resistant granules are relaxin and Velardo et al ( 53) while speculating that the metrial gland might furnish such a factor have associated the gland with a continuation of luteal function in the ovaries Dickson and Bulmer ( 61) have also attributed a possible luteotropic function to metrial gland cells

The present paper reports the localization and describes the differentiation of metrial gland cells and their precursors in the mouse uterus from two and three quarters to seven and one quarter days post coitum The time of acquisition of glycogen by the metrial gland cells and other glycogen-containing cells of the mouse uterus and of the embryo is also given A detailed account of concomitant changes in other endometrial cells may be found in Smith ( 66)

## MATERIALS AND METHODS

*F1 females* from crosses of mice of either the Balb or J strain to animals of various mutant stocks were exposed to

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## MATERIALS AND METHODS

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F1 males from the same cross in the eve ning and checked for vaginal plugs the following morning Mating was arbitrarily assumed to have occurred at 1 00 AM of the day the plug was found and the fe males were killed at intervals from one half to seven and one-quarter days after wards Each female had produced one or two litters before being sacrificed The rate of embryonic development was in com plete accord with that reported by Snell (41)

To demonstrate basophilia the uteri were fixed in Carnoy s fluid and stained with Azure B (Flax and Himes 52) Gly cogen was determined by the PAS method All uteri for the latter study with the ex ception of those of the three and one half day stage were fixed in Lison s Gendre fluid cooled in an acetone $CO_2$ mixture Those for the three and one half day stage were fixed in Carnoy s fluid Control sec tions were subjected to amylase digestion prior to the PAS procedure

## RESULTS

### *Metrial gland cells*

As early as two and three fourths days after mating in uteri stained with Azure B scattered cells with basophilic cyto plasm stand out against a background of palely stained stellate endometrial stro-mal cells The basophilic cells are of two usually distinct types one plasma cells *the other* cells thought to be the precur sors of the metrial gland cells The sup posed precursor cell unlike the plasma cell is round and its cytoplasm shows no lightly stained patch or halo In addition its diffusely stained nucleus contains 1 or 2 strongly basophilic irregularly shaped nucleoli often attached to one side of the nuclear membrane a feature which fur ther distinguishes this cell from the plasma cell (fig 2) As shown in figure 1 the size of the round cells seen at 65 hours varies considerably *The smallest* is the size of a large lymphocyte while the largest has both a larger nucleus (fre quently kidney shaped) and a wider rim of cytoplasm The nucleoli enlarge as cell size increases *Much of the* basophilic material in the cytoplasm of the largest of these round cells even at 65 hours is in the form of thick filaments and in later stages of pregnancy when still larger cells of this type are present the filaments in them are more widely separated giving these cells a very distinctive appearance The larger round cells with basophilic filaments will henceforth be called met rial gland cells the smaller round cells dark cells Both dark and metrial gland cells are found in small numbers through out the stroma under the luminal epi thelium and around the glands at 65 hours after mating They are often concentrated near the enlarging endometrial capillaries characteristic of this stage of pregnancy

From 65 to 96 hours after mating the total number of metrial gland and dark cells the proportion of metrial gland to dark cells and the maximum size of the metrial gland cells increases steadily It is at 96 hours that blunt processes from the trophoblast are first seen within the uterine epithelium in the mice used in this study

At four and one quarter days after mat ing the dark and metrial gland cells are found principally in the mesometrial re gion of the implantation site although there are a few in the antimesometrial decidua Surprisingly there are also many large vacuolated metrial gland cells pres ent around the lumen in the area between implantation sites (fig 3)

As late as six days after mating or when the embryo is well implanted ex tremely vacuolated metrial gland cells are still seen occasionally between the very large antimesometrial stromal cells The number in this region is negligible though when compared to that in the mesometrial decidua where medium sized metrial gland cells and large dark cells are particularly numerous around the mesometrial end of the remaining lumen and in the region of the sinusoid wings A few large met rial gland cells can still be found in the interdecidual regions

*By seven and one quarter days the re* gion around the mesometrial lumen and destined to form the mid portion of the decidua basalis has increased in size markedly At this time it contains very large numbers of medium sized metrial gland cells (fig 4) many of which in sections stained with hematoxylin can be

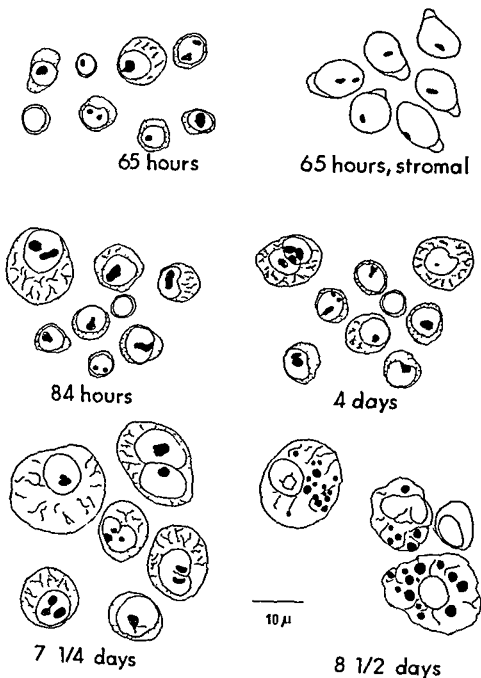



Fig 1 Representative metrial gland and dark cells found in the mouse endometrium from two and three-quarters *to eight and* one half days aft r mating Also representative stromal cells from around the mesometrial lumen at two and three-quarter days Camera lucida drawings made at 3,200 × and reduced to 1,280 ×

seen to be lying on the basement membrane of the swollen capillaries The endothelial lining cells of the latter are now basophilic and enlarged A few very large metrial gland cells and very many of the smaller ones surround the highly developed sinusoids laterally At seven and one quarter days metrial gland cells have disappeared from between the implantation sites

In hematoxylin stained sections of eight and one half day pregnant uteri large metrial gland cells with large cytoplasmic granules are found in the decidua basalis and occasionally one lies free in the maternal blood spaces between the giant trophoblast cells It is at eight to eight and one half days that the allantois first makes contact with the ectoplacental cone in the mice used in this study

In summary small round basophilic cells which seem to be related to large lymphocytes through a series of transitional forms are present in the endometrium at 65 hours after mating Similar round basophilic cells are found at all subsequent stages examined and a continuous series of transitional forms links them to mature metrial gland cells in the oldest stage examined (fig 1)

*General glycogen localization*

At three and one half days after mating much glycogen is present in the lumens of the uterine glands and small granules of glycogen are found in the apical cytoplasm of cells of the uterine glands near the periphery of the endometrium No glycogen is demonstrable in the cells of the luminal epithelium or in the fusiform stromal cells However an occasional round cell in the stroma having a spherical or kidney shaped nucleus contains a few cytoplasmic granules adjacent to the area where the nucleolus apposes the nuclear membrane (a metrial gland cell?) Some oval cells (plasma cells?) and what appear to be polymorphonuclear leucocytes are also PAS positive In the three and one half day blastocyst PAS stainability is high in the trophoblast and somewhat less intense in the inner cell mass

In the most developmentally advanced four and one quarter day stages embryonic glycogen has disappeared but cells in a cup shaped area of decidua antimesometrial to the embryo (part of the primary decidual zone of Krehbiel 37) contain small granules By four and three quarter days cells in the lateral primary decidual zone are also reactive

At five and one half days cells in the antimesometrial and lateral primary decidual zone are no longer PAS positive while those in the primary decidual zone adjacent to the disintegrating luminal epithelium mesometrial to the embryo are This is the area called glycogenic area by Krehbiel (37) and the basophilic cell zone by Smith (66) By six days an extremely heavy deposit of glycogen is present in the basophilic cell zone In addition glycogen begins to reappear in the embryo at this time for 2 or 3 PAS positive cells are found within the ectoplacental cone of some of the embryos Other structures also PAS positive are the apices of the vacuolated endoderm cells covering the egg cylinder Reichert s membrane and a few giant trophoblast cells In all these however the reaction is not eliminated by amylase digestion

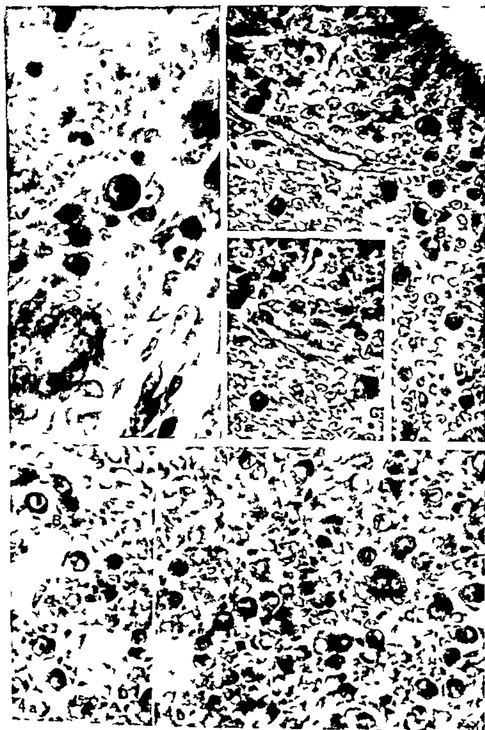
At six and three quarter to seven days after mating the glycogen reaction has spread to the fusiform cells of the sinusoid region lateral and mesometrial to the basophilic cell zone Many of the metrial gland cells in this region and in the area of small cells surrounding the mesometrial lumen also contain glycogen the granules in them however are fewer and much smaller than those in other glycogen bearing cells Glycogen is now also present in small mononuclear cells located between

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Fig 2 The large round cell in the center of the field is a dark" cell To the left of it are three plasma cells From a uterus taken three and one half days after mating Azure B 10 μ 1 000 ×

Fig 3 Dark and small metrial gland cells in the interdecidual region at four and one half days Note the height of the luminal epithelium in this region at this stage a and b are two levels of the same section Azure B 10 μ 500 ×

Fig 4 Dark and metrial gland cells in the compact zone at seven and one-quarter days after mating a and b are two levels of the same section Azure B 10 μ 500 × A a small dark cell B large dark cells C cells with basophilic filaments D a highly vacuolated cell


4a
4b

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treated with estrogen and relaxin or estrogen alone

*Function of metrial gland cells*

It is of course frequently the case that pattern formation during differentiation is the result of loss of cells in some regions and their continued differentiation in others and this may account for the presence of metrial gland cells throughout the endometrium as early as 65 hours after mating in the inter-decidual sites up until six and one half days in the decidua basalis until about 14 days and in the metrial gland until term Nevertheless their continuous presence in some portion of the uterus from 65 hours until term and their localization around the vascular channels may indicate that they subserve some long term function other than supplying glycogen to the embryo Several such long term functions can be and have been suggested For example if metrial gland cells do indeed arise from lymphocytes or thymocytes a possible role in the suppression of the maternal immune reaction might be looked for Both Simmons and Russell (62) and Edidin (64) have data which suggest that the mouse trophoblast in immunologically competent in very early stages perhaps even as early as seven days after mating

Long term relaxin like activity is another possibility (Velardo et al 53 and Wislocki et al 57) The data of Bloom et al (58) however argue against the idea that relaxin is found in the metrial gland cells since their extract of metrial glands taken at 21 days of pregnancy did not affect myometrial motility of the rat uterus while an ovarian extract did Kroc et al (59) also found a high level of relaxin activity only in ovaries of pregnant rats and mice and not in placentae or uteri

A luteotropic function must also be considered and the acidophilic granules have been linked to this hormone by Dickson and Bulmer (61) Furthermore both Velardo et al (53) and Melampy et al (64) have presented evidence that the greater the amount of decidual tissue in rats with induced deciduomata the longer the duration of pseudopregnancy Melampy et al however suggest that the "transformation of endometrium into decidual tissue reduced luteolytic activity" rather than increased luteotropic activity They also report that "prolactin injected daily with decidual tissue in one horn was sufficient to override the luteolytic activity of the non traumatized horn

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The changing pattern in decidual glycogen localization found in this study in cells other than metrial gland cells seems identical in the main to that described by Krehbiel (37) for the rat although the timing differs in the two species Glycogen is present only in the antimesometrial cells of the primary decidual zone at four and one-quarter days after mating in the mouse at six days in the rat The glycogen reaction has spread to the cells of the lateral primary decidual zone by four and three-quarter days in the mouse and by six and one half days in the rat Glycogen has disappeared from these regions and is present in the glycogenic area only by five and one half days in the mouse and by seven and one half days in the rat In the implanting mouse blastocyst glycogen disappears shortly before glycogen is visible in the decidua and it reappears in the central portion of the ectoplacental cone when the sinusoid regions of the mesometrial decidua are acquiring large amounts Bridgman (48a) says that glycogen can be found in the cone of rat embryos by the sixth day

In the mouse in the subepithelial antimesometrial and lateral primary decidual zone cells and in the mesometrial decidual cells next to the degenerating epithelium lining the decidual crypt the acquisition of glycogen is preceded by a sharp diminution in basophilia (Smith 66) The latter apparently follows the occlusion of the subepithelial capillaries as the result of the great increase in size of the endothelial cells in this region The cells with reduced basophilia then become compressed and flattened and disappear by about the 6th day Presumably they are phagocytized by the differentiating trophoblast giant cells although occlusion of the capillaries must play a part in their breakdown It seems reasonable to assume that the glycogen of these glycogen containing cells

the basal layer of the uterus and the sinusoid wings and between the basal layer and the most mesometrial cells of the antimesometrial decidua From their location this new type of glycogen containing cell appears to be in a portion of the fibrinoid capsule of Bulmer and Dickson ( 61) Many cells within the ectoplacental cone are now reactive

## DISCUSSION

### *Origin of metrial gland cells*

Almost as many types of cells have been proposed as metrial gland cell precursors as there have been investigators describing them Jenkinson ( 02) and Gérard ( 27) who studied metrial gland cells in the mouse suggested that they were derived from the fusiform stromal cells Baker ( 48) and Velardo et al ( 53) proposed an origin from mesometrial mesenchymal cells in the rat Selye and McKeown ( 35) stated that almost any mesenchymal cell in the rat endometrium myometrium or the region of the mesometrial triangle might be able to dedifferentiate come to resemble a small fibroblast then redifferentiate into a typical metrial gland cell Lewis ( 24) who studied Hofbauer cells in the early human villus said there were transitional forms between them and mesenchymal cells On the other hand the variation in size of the round and oval ones down almost to the size of white blood cells suggests the possibility that they may arise from white blood cells that have wandered in the tissue spaces Hofbauer cells appear to be morphologically identical to metrial gland cells They too contain glycogen show a residual reaction with the PAS technique after digestion or contain occasional droplets which stain with hematoxylin (Wislocki and Padykula 61) It was similarities such as these which led Dickson and Bulmer ( 61) to suggest that some metrial gland cells of the decidua basalis of the rat might be derived from the trophoblast

Several of the observations reported here suggest the possibility that a cell of the lymphocytic series may be the precursor cell First there appears to be a continuous series of transitional forms from the larger lymphocytes to the smallest basophilic dark cells and from the dark cells to mature metrial gland cells Second both lymphocytes and the smaller dark cells are found together in the tissues near the enlarging capillaries at 65 hours and at later stages the basophilic cells are particularly numerous near the sinusoids of the decidua basalis and metrial gland Finally the great developmental potentiality of lymphocytes or thymocytes is suggested by their recently demonstrated polymorphism (Murray and Woods 64 for example) Fortunately the method used by these authors provides a way of testing this hypothesis

The present study does not exclude any of the other proposed hypotheses of the origin of metrial gland cells but lends more weight to some than to others For example as is evident from figure 1 the nucleus of the stellate mesometrial stromal cells at 65 hours is larger and the nucleoli are smaller than those of the smallest basophilic cells suggesting that the smallest basophilic cells are not merely rounded up stromal cells It is less easy to reject the possibility that metrial gland cells may be escaped endothelial cells or perhaps primitive reticular cells from the capillary walls There does seem to be an association in time between the enlargement and appearance of basophilia in the endothelial cells and the presence of metrial gland cell precursors This association is especially evident at 65 hours when metrial gland cell precursors are first seen and at seven days when they increase in number markedly in the central portion of the decidua basalis

No matter what their origin however on the basis of the time of their first appearance it seems reasonable to assume that the stimulus which sets off the differentiation of the metrial gland cells may be the same stimulus which triggers differentiation of the uterus into an organ responsive to the stimulus for decidualization In this regard it is interesting that Hall ( 60) found large vacuolated cells containing both glycogen and PAS positive diastase resistant granules in the stroma of ovariectomized virgin mice treated with progesterone and estrogen and not in those

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serves as a source of energy for the developing embryo although PAS positive saliva-digestible material is not found in the trophoblast cells or in the endoderm covering the egg cylinder at this stage

Jollie and Bencosme (65) followed the fate of what appear to be similar glycogen containing cells in the rat from just before the induction of decidualization (day 0) until five days after trauma They report that lysosomes first appear at day 2 and are present until day 5 However they also report that glycogen granules were not identified in the cells they examined before day 4 Day 4 would seem to correspond to the 8th day post coitum in a typical implantation

In contrast to the situation with respect to the primary decidual zone cells the glycogen containing cells in the sinusoid regions and in the basophilic cell zone (the glycogenic area) just outside the mesometrial portion of the implantation zone do not lose their basophilia before acquiring glycogen (Smith 66) The reason for this difference is unclear but the capillaries in the latter two regions do not become occluded and these glycogen bearing cells are much longer lived (Jenkinson 02)

Finally although there are many similarities in glycogen localization in rats and mice there are also differences One of the latter may help explain why there has been little doubt expressed concerning the origin of some of the glycogen-containing cells of the mouse ectoplacental cone and placenta (Jenkinson 02) although this has not been the case for the rat (Bridgman 48b Bulmer and Dickson 60) For instance plate 1 figure 1 of Padykula and Richardson (63) and their description of glycogen in the 8 day ectoplacental cone and adjacent decidual cells indicates that these glycogen areas are closer to each other in the rat than they are in the mouse A comparison of the glycogenic area of the decidua and of the cone in Krehbiel s (37) diagrams for Day 8 and 9 and in Smith s (66) figures 9 and 10 shows this also The existence of a wider band of maternal cells free of glycogen between the maternal and fetal glycogenic areas in the mouse at the time the cone is aquiring glycogen may be one reason the separate origins of the glycogen bearing cells in the maternal and fetal glycogenic areas of the mouse seems quite clear Perhaps the propinquity of these same areas in the rat has also contributed to the uncertainty over the relationship between the metrial gland cells of the decidua basalis and similar cells in the trophoblast in older stages of pregnancy (Dickson and Bulmer 61)

## ACKNOWLEDGMENTS

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# Studies on Sperm Survival Mechanisms in the Female Reproductive Tract of Hibernating Bats

## I CYTOLOGY AND ULTRA STRUCTURE OF INTRA UTERINE SPERMATOZOA IN *MYOTIS LUCIFUGUS*[1,2]


WILLIAM A WIMSATT PHILIP H KRUTZSCH[3] AND LEONARD NAPOLITANO[4]

*Division of Biological Sciences Cornell University Ithaca New York and Department of Anatomy University of Pittsburgh School of Medicine Pittsburgh Pennsylvania*



ABSTRACT Among mammals prolonged survival of spermatozoa in the reproductive tract of the female (circa 7± months) is known in only a few species of hibernating bats but its physiological basis remains obscure Presumably survival involves special physiological adaptations of the spermatozoa the uterus or both We have initiated studies of sperm survival mechanisms in the hibernating bat *Myotis lucifugus* involving analysis of the cytology and fine structure of intra uterine sperm the morphology and histochemistry of sperm uterus interactions and selected biochemical parameters of the reproductive tract This paper summarizes our findings concerning the morphology of epididymal and intra uterine spermatozoa and emphasizes those aspects not detailed by others The microscopic organization of epididymal sperm is described in detail with particular attention to peculiarities of the head middle piece and cytoplasmic droplet The latter contains amylase resistant PAS+ granules and acid phosphatase both possibly of lysosomal origin Most ultrastructural features elucidated by Fawcett and collaborators are confirmed but additional details concerning the head post nuclear sheath origin of axial filament complex and the outer coarse fibrils are provided A functional interrelationship between coarse fibrils 3 and 8 and the longitudinal columns of the fibrous sheath is also postulated

Structural differences between epididymal and uterine sperm are minor suggesting that uterine environmental factors are most important for sperm survival

A new basis is suggested for the apparent helical arrangement of mitochondria in the middle piece In *Myotis* sperm the configuration is illusory being the result of an orderly alternation in the staining of the serially arranged mitochondrial pairs We suggest that the "patterned" staining of mitochondria may reflect sequential cyclic functional alterations of significance in the initiation and/or coordination of the geometrically oriented tail movements


The unusual reproductive habits of hibernating vespertilionid and rhinolophid bats have long been known but many aspects of their physiological regulation remain obscure A major unsolved problem concerns the mechanism of sperm storage and survival in the female reproductive tract where the cells may retain their viability for as long as 5–7 months (Mat thews 37 Wimsatt 44) Prolonged storage of viable spermatozoa within the female tract is by no means novel among invertebrates or even lower vertebrates but among mammals the phenomenon observed in bats is unique and is therefore of great theoretical interest Earlier workers have variously proposed with scarcely any direct evidence that lowering of body temperature mechanical or chemical inhibition of motility elevated CO levels secretion of nutrients and other


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[2] Shortly aft r an origin l draft of thi article had been compl ted, the splendid study of Faw ett and Ito ( 65) on the fine structure of bat *sperm was pub*lished The overl p between thei observ tions and ours was sub tanti l and we h ve felt obliged to revise our fine tructu ection to void publi hing f ankly repetiti e m terial. The pr sent section on ultr structure is a subst ntially shortened version of the origin l. We obviously could not eliminate all redund nt obs rvations and till achiev a coherent account, but those retain d for th most part complement differ from or c nfirm where thi w deemed desir ble the bs rv tions of Fawcett and Ito Furthermore our primary objective has been and i to determine th bases of sperm longevity in the uterus and our observ tions of fine tru ture are focused m inly on intr uterine sperm, whereas those f Fawcett and Ito d alt wholly with un j ulated sperm from th epididymis Ch racterization of the fine tructure of urviving sperm *in utero* as comp red with those still within the epididymis is essential to the *achievement* of our main objective The section summ rizing our microscopic nd histochemical observations has not been ltered from th original sin these observ tions are not dupli ated in the study of Fawcett and Ito.


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derson (63) Control Sections (cryostat following cold formalin fixation) of prostate and liver were run simultaneously with the sperm preparations as were also comparable preparations in which enzymic activity had been inhibited by sodium fluoride All tests of sperm were carried out on fresh cell suspensions from the epididymis Thick sperm suspensions were activated in Locke s fluid for 15 minutes The sperm were then smeared on slides rapidly dried and fixed briefly in cold formal-calcium They were then quickly rinsed in distilled water and immersed in the incubating medium The control slides were treated similarly except they were exposed to a 0 1% NaFl solution for 20 minutes at 60 C before being placed in the incubating medium In other instances the fresh sperm suspensions were introduced directly into the incubating media (or in fluoride solution followed by incubating medium) and only after their removal from the latter were spotted on slides dried fixed dehydrated and mounted Comparable results were obtained with both procedures

We have adopted here the terminology for sperm cell components recommended by Fawcett (66) and are grateful to Dr Fawcett for his kindness in allowing us to examine his paper in proof

The expert technical assistance of Mr Anthony Guerriere and Dr Mona L Coetzee in the careful execution of these procedures is also gratefully acknowledged

## MICROSCOPIC OBSERVATIONS

### *Pre-ejaculatory spermatozoa*

In order to establish base lines for delineation of possible morphological changes in surviving intra uterine spermatozoa we have examined cell suspensions and smears of sperm expressed from the caudae epididymidis of male bats killed directly from the hibernating state in the months December through April Most of the structural details to be reviewed are observable in figures 3 to 8 inclusive

The only variable observed in sperm samples collected on different dates involved the ctyoplasmic droplet Droplets were an obvious component of the majority of sperm from two males killed in mid December a single specimen each in January and February and in three April specimens On the other hand droplets were scarce in four December specimens and were absent in approximately 20 additional bats killed in January February March and April In *Myotis* spermatogenic activity ceases in September so epididymal sperm in hibernating animals are products of the previous summer s activity Loss of the cytoplasmic droplet attends sperm maturation and the tendency for the droplets to disappear in late winter animals is perhaps not surprising Why they should persist in sperm from April animals when they were already lost in others killed earlier in the season is not clear Dormancy (as in hibernation) is characterized by a drastic reduction of metabolic functions and body temperature with an attendant curtailment of cellular activities of all sorts including probably sperm maturation in the caudae epididymidis (Wimsatt 60) But hibernation in bats is not a continuous process spontaneous arousals do occur and probably with individual variations in frequency During these periods metabolic activity and body temperature return to normal activity levels and cell metabolism is increased The chronological rate of sperm maturation in the epididymis would likely be influenced by the total elapsed time the individual animal had been active during the winter months Presumably the April specimens whose sperm retained cytoplasmic droplets were those whose arousals (spontaneous or induced by disturbance) had been of minimal frequency and duration

In most epididymal sperm regardless of the month of collection the droplet when present was attached distally on the middle piece just cephalad of its junction with the principal piece Typically it is eccentric globular and rarely constricted at its attachment Less frequently it is annular forming an obtuse spindle like swelling about the end of the middle piece which passes through its center (figs 3 9) In a small proportion of spermatozoa examined especially from the first two December specimens mentioned above droplet material was located at higher levels in the middle piece even as far forward as the neck region in some instances Often in

factors singly or in combination somehow support sperm longevity The alternatives that bat spermatozoa may be inherently specialized or that the adaptive capability may have evolved on both sides during evolution of the hibernating habit have been scarcely considered

The more dynamic aspects of the sperm survival mechanism can presumably only be resolved through application of appropriate physiological and biochemical procedures but delineation of any special morphological adaptations or interrelations between stored sperm and the tissues of the female tract may be indispensable for a complete understanding of the phenomenon The morphology of stored spermatozoa and of sperm uterus relationships has not been systematically explored in any hibernating bat A study has been undertaken principally in *Myotis lucifugus* a common hibernating vespertilionid bat of the eastern and central United States Observations will be presented here on the microscopic and ultrastructural organization of epididymal and intra uterine sperm The sperm uterus relationship and certain biochemical findings will be analyzed in later reports

## MATERIAL AND METHODS

Specimens of the little brown bat (*Myotis lucifugus lucifugus*) were collected from natural populations hibernating in caverns in New York Pennsylvania New Jersey and West Virginia throughout the natural hibernating period In the laboratory all specimens were maintained in an artificial hibernaculum under controlled temperature and humidity until sacrificed The bats were killed by cervical fracture while still torpid and the cauda epididymides and uteri were immediately removed for fixation Materials intended for analysis by electron microscopy were handled as follows the fresh tissues were cut into blocks of approximately 1 mm³ and immersed in cold phosphate buffered osmium tetroxide (1%) for 1 to 2 hours Following rapid dehydration in increasing concentrations of cold ethanol the tissue blocks were embedded in Vestapol W Sections were cut on a Huxley microtome and those displaying gold interference colors were mounted on grids and stained by the lead hydroxide tecchnique of Karnovsky (61) Electron micrographs were obtained in a Phillips EM 200 at initial magnifications of 2 000 to 34 000

*Materials intended for light microscopy* were variously fixed in 10% neutral formalin Bouin Helly and chilled Zenkers and Rossman s fluids For the most part fixation was accomplished by immersing the small organs in toto but in several instances the cornual extremities of the uterus or the oviducts were separated and fixed independently in cold reagents By using torpid animals and chilled fixing solutions it was hoped to minimize sperm mobilization and displacement within the organs Following dehydration in ethanol and clearing in cedar oil dioxane or chloroform the tissues were embedded in paraffin cut at 5 to 10 μ on a rotary microtome and mounted on glass slides Staining procedures routinely applied included Harris hematoxylin and eosin iron hematoxylin Masson trichrome eosin and methylene blue (methylene blue in buffered solution at pH 6 0–6 5) and the periodic acid Schiff (PAS) procedure with or without hematoxylin counterstain

Preparations of whole epididymal and uterine sperm were made by opening and stripping the organs in a small drop of distilled water or saline The resultant sperm suspensions were diluted to the desired density with additional water taken up in a Wintrobe pipette and spotted on glass slides resting on a warm plate The preparations were fixed immediately after drying in 10% formalin cold Rossman Carnoy Regaud Helly formal calcium Bodian or absolute ethanol and ether rinsed and again dried They were then variously stained in hematoxylin and eosin iron hematoxylin PAS and hematoxylin Bodian protargol Holmes (43) silver stain Feulgen Masson trichrome and Baker s acid hematein Those fixed in Regaud were postchromed in 3% potassium dichromate for 3 to 8 days at 37°C and stained in Altmann s aniline acid fuchsin methyl green phosphotungstic acid hematoxylin and Sudan black B

Sites of acid phosphatase activity were visualized by the modified Gomori lead sulphide and Burstone *azo-coupling* procedures recommended by Barka and An

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such cases the droplet was flattened and spread out along one side of the middle piece, being the more flattened nearer the head Only a special staining characteristic (described below) enabled this extremely flattened droplet material to be identified with certainty

The cytoplasmic droplets are generally resistant to staining They are usually more easily observed with phase-contrast optics than in stained light microscope preparations They do demonstrate a feeble acidophilia similar to that of the general cytoplasm of the middle piece but basophilic components were not demonstrable In one respect, however the staining of the droplets is unique they react intensely and selectively to PAS and the staining is unaffected by the previous action of salivary amylase so it is not attributable to glycogen In the more flattened droplets of younger spermatozoa the reactive material is discrete and granular, and serves as a selective marker for the identification of presumptive droplet cytoplasm in the younger cells In older spermatozoa with distal globular or annual droplets the PAS reaction is more homogeneous permeating the cytoplasm of the droplets except centrally, where a clear area surrounds the mitochondrial sheath (fig 3) The situation in younger sperm suggests that PAS reactivity is associated with discrete but exceedingly minute cytoplasmic organelles a discrete morphological basis for the denser more homogeneous reaction in droplets of older sperm was not discernible at the microscopic level The PAS reactivity of the cytoplasmic droplet in *Myotis* sperm is the more striking in that no other constituent is stained not even the acrosomal cap (see below) It is not unique however for Bishop and Walton ( 60) cite an observation by Hancock that droplets in other mammalian sperm (not identified in citation) are PAS positive and Cavazos and Melampy ( 54) call attention to PAS reactive materials in the droplets of bull ram and boar sperm Possibly the phenomenon is general in mammals

Another histochemical peculiarity of many cytoplasmic droplets was the presence of acid phosphatase as revealed by the Gomori lead sulphide method (fig 13) The staining was never uniform, for in any given preparation seldom more than a third of the droplets present showed a positive reaction a result which is at least consistent with the well known vagaries of the Gomori procedure In reactive droplets the reaction product tended to be concentrated peripherally, while the central area surrounding the mitochondrial sheath remained unstained A positive reaction was also observed in many of the flattened non terminal droplets of presumably younger sperm It is thus seen that the distribution of enzymic activity coincides with that of the PAS reactive materials previously mentioned Control slides treated with NaFl which inhibits acid phosphatase activity were always uniformly negative We were unable to demonstrate acid phosphatase unequivocally in sperm by means of the Burstone azo coupling procedure, even though simultaneously processed control sections of prostate and liver gave strong positive reactions Possibly the explanation lies in the lower contrast or optical transparency of the reaction product where present in minute quantities, or perhaps to a lower sensitivity of the azo-coupling procedure

The head of the spermatozoon in *Myotis* is somewhat elongate the length being slightly greater than twice the width (figs 3–6) The overall length is less however than it appears to be in the sperm of the greater horseshoe bat (*Rhinolophus ferrum-equinum*) depicted in phase contrast and ultraviolet micrographs by Bishop and Walton ( 60) The principal difference resides in a greater elongation of the acrosomal cap in *Rhinolophus* In other respects the heads of the spermatozoa of these two hibernating (although not closely related) bats are more or less similar At cursory glance the head of an unsectioned *Myotis* sperm resembles a cylinder with straight sides which converge ever so slightly posteriorly In reality it is flattened only slightly at the base but progressively more so anteriorly so that when viewed on edge it presents a triangular or wedge shape (figs 1 17) In normal (flat) aspect the anterior end of the nucleus is rounded and we have determined by Feulgen staining and phase-contrast microscopy that it extends nearly to the tip

of the acrosomal cap This appears not to be so in *Rhinolophus* sperm (cf figs of Bishop and Walton 60) where the cap extends substantially beyond the nuclear apex In *Myotis* the base of the nucleus which is slightly ellipsoidal in section profile is deeply invaginated and into this oval depression or implantation fossa" inserts the short and apparently constricted neck piece (figs 1 4 17) The region above the curvilinear margin of the fossa shows up in smear preparations as a dense basophilic band it is selectively stained by silver in the Bodian protargol technique (fig 7) and less conspicuously by the Holmes procedure (fig 8)

The detailed morphology of the acrosomal cap in *Myotis* spermatozoa is not easily visualized microscopically for it is simple in form extends only slightly beyond the nuclear apex and is both optically transparent and remarkably resistant to staining Fortunately these difficulties are circumvented in electron micrographs to which we shall allude shortly Nevertheless the presence of the cap is indicated microscopically by a progressive "fading out of the stained nucleus anteriorly beginning about two thirds of the way back from the nuclear apex (figs 3–6) This point coincides with the posterior margin of the acrosomal cap and is frequently indicated by a stainable but usually diffuse band which extends across the nucleus at right angles to its long axis this band was most consistently revealed by iron hematoxylin but is also apparent in acid phosphatase (Gomori procedure) preparations (fig 13) These observations suggest that the acrosomal cap is thin posteriorly and thickens progressively nearer its apex There are no lateral extensions or wings and the smooth anterior margin of the cap reproduces the uniformly curved profile of the nuclear tip It is at present unknown whether the acrosomal materials of transforming spermatids in the testis of *Myotis* are PAS reactive (as in rat and other rodents Clermont and Leblond 55 Leblond and Clermont 52) but the acrosomal cap material itself was totally unreactive in smears of epididymal and intrauterine sperm regardless of the fixation employed

The middle piece is perhaps the most diagnostic segment in *Myotis* spermatozoa for it is unusually thick and prominent (figs 3 4) Constituents easily recognized in stained smears include a central filament passing through the length of the segment a conspicuous mitochondrial sheath having the apparent form of a helix and a prominent cytoplasmic sheath and limiting membrane An undulant or twisted profile of the middle piece which we have occasionally seen and which has been mentioned or figured by others (e g Hirth 60 Fawcett and Ito 65) is presumably a shrinkage artifact for it is not observed in well fixed material The mitochondrial component was generally apparent in Masson or iron hematoxylin preparations and even in sperm stained solely with eosin but was always revealed with greatest clarity by special mitochondrial stains such as Altmann s aniline acid fuchsin methyl green or phosphotungstic acid hematoxylin after Regaud fixation and prolonged post-chromation (fig 4) It is also demonstrated effectively by Baker s acid hematein and Sudan black Typically at lower magnifications and even under oil immersion in less than perfect preparations the mitochondria collectively display the continuous coil like arrangement so often referred to in the classical descriptions of mammalian spermatozoa it can readily be appreciated how cytologists may have been misled into believing that the mitochondria fuse end to end to form a single or double helically wound strand within the middle piece That fusion does not actually occur at least in the sperm of higher mammals has now been well demonstrated by electron microscopy and *Myotis* sperm are no exception

While the length of the middle piece is only slightly greater than three times the head length the principal piece is appreciably longer being approximately eight times the head length Hirth (60) gives the following average figures for *Myotis lucifugus* sperm head 4 3 μ middle piece 12 7 μ principal and end pieces together 38 μ for a total cell length of approximately 55 μ There is no abrupt reduction in girth at the transition between middle piece and principal piece but the latter does begin here to taper gradually and uni

formly to its end (figs 3 4 5 8) In stained preparations however the transition appears abrupt for among the stained elements of the middle piece only the central filament continues on into the principal piece In most stained microscopic preparations the boundary between principal and end pieces was not visualized although they are readily separable by use of phase contrast optics The principal piece is further distinguished from the middle piece by its greater apparent density and enhanced acidophilia (fig 5) These features in combination with the greater difficulty encountered in visualizing the central filament within the principal piece are presumably related to the presence of the fibrous sheath which is restricted to this segment of the flagellum It was found that the Holmes ( 43) silver nitrate procedure (for nerve fibers) stained the axial complex of the middle piece and the fibrous sheath of the principal piece in a highly selective manner (fig 8) An unusual feature observed only in the Holmes preparations was an intense blackening of a short spatulate segment at the distal end of the principal piece The reaction was consistent and characteristic A morphological or other basis for it was not discernible in other types of preparations and its significance is unknown Its constancy in the Holmes preparations suggests however that it is not an artifact

The so called annulus (end ring ring centriole) and proximal centriolar bodies were not resolved in the stained smears The centriolar complex lies within the implantation fossa at the nuclear base and is not easily seen in stained whole spermatozoa The annulus situated at the end of the middle piece either resemble the mitochondria in its staining reactions or remains unstained for it is not clearly distinguished in these microscopic preparations

*Abnormal epididymal spermatozoa*

Abnormal spermatozoa were observed occasionally in the epididymal smears and resembled for the most part common abnormalities described in other species Most of them involved larger than normal heads with accompanying grossness of the middle piece and anterior or posterior duplication of parts (fig 6) Decapitate sperm were also noted, but they could have been casualties of the technical procedures employed In most of the abnormal sperm terminalization of the cytoplasmic droplet had not occurred most often the droplet cytoplasm was flattened and situated proximally on the middle piece but was easily discernible in PAS preparations because of its content of reactive granules

*Intra uterine spermatozoa*

The study of uterine sections and of sperm suspensions and smears from the uterus of hibernating animals failed to reveal any striking alterations in sperm morphology over the organization of epididymal sperm just described except for the absence of the cytoplasmic droplet In no case was a droplet found on intra uterine sperm nor is this surprising for it is normally jettisoned by the sperm of other mammals at ejaculation if not sooner There was perhaps a slight tendency for the mitochondrial helix of intra uterine sperm to be less easily stained than in those from the epididymis but its general organization and that of the other head and tail components were not obviously altered over the epididymal condition If there are subtle morphological changes in bat sperm associated with their prolonged intra uterine life they were not detectable by these light microscopic procedures

ELECTRON MICROSCOPIC OBSERVATIONS

The fine structure of epididymal sperm in *Myotis lucifugus* was partially characterized in several earlier papers by Fawcett and collaborators (Fawcett 58 62 Telkka Fawcett and Christensen 61) and recently in more comprehensive fashion by Fawcett and Ito ( 65) Our observations duplicate in most respects the observations of Fawcett s group Hence we shall restrict our description of fine structure of bat sperm to those observations which extend clarify differ from or confirm in certain instances where this is deemed useful the findings of the Harvard group We assert at the outset that at the ultrastructural as well as at the microscopic level we have observed no significant organizational differences between surviving intra uterine sperm in hibernating females and those

from the epididymis other than the ab sence of ctyoplasmic droplets and a slight reorganization of the neck

*The cytoplasmic droplet*

The cytoplasmic droplet has been little studied in reference to its fine structure Bloom and Nicander (61) examined the droplets of bull ram rat and rabbit They record differences in droplet morphology not only as between species but also in rela tion to the location of sperm within the epi didymal tubule and the progressive phases of sperm maturation Fawcett and Ito (65) provide a brief description of the cytoplasmic droplet in epididymal sperm of *Myotis* and while our observations accord with theirs a somewhat fuller characteriza tion than they have provided seems war ranted Examples of terminalized (on the middle piece) droplets in epididymal sperm are depicted in figures 9 to 11 inclusive The droplet is bounded externally by a single dense membrane (plasmalemma) continuous with that enclosing the mid dle piece above and the principal piece be low Where the membrane reflects from the caudal margin of the droplet onto the principal piece it passes over and is firmly adherent to the outer surface of the dense annulus" The abundant cytoplasm of the droplet directly encloses and infiltrates the mitochondrial sheath and axial fila ments which pass through it The matrix is finely granular relatively homogeneous and free of ribosomes and lipids

The principal cytoplasmic inclusions are numerous double layered membranous sac cules most of which present circular or semi-circular profiles in section view The matrix invested by the membranous wall of these semi vesicles resembles and com municates widely with the surrounding general cytoplasmic matrix The three di mensional form of many of the semi vesi cles appears analagous to that of a hollow rubber ball in which one hemisphere is completely invaginated into the other with or without elongation and "pursing of the opening The continuous circular profiles are presumably sections which miss the opening of the invaginated globes Some of the flattened saccules are less cupped than the majority and a few are adjudged to be semi tubular or ribbon like in three dimensional aspect Scattered among these larger double walled elements are variable numbers of smaller circular vesicles and tubular structures delimited by a single smooth surfaced membrane A few similar bodies are scattered randomly between the mitochondria of the middle piece All of these structures may or may not be com ponents of a single membranous system Since the membranes are non granulated and we lacked spermatid transformation stages for comparison we are uncertain as to their nature but they most probably are derived from Golgi lamellae ER vesicles of the spermatid (a conclusion reached also by Fawcett and Ito 65) or possibly lysosomes In the more globate cytoplas mic droplets there is a distinct tendency for the membranous inclusions to be dis posed peripherally adjacent to the outer limiting membrane leaving a zone of free cytoplasm immediately surrounding and within the mitochondrial sheath These displaced bodies are visible as a dark pe ripheral band of granules in good phase contrast preparations of living sperm

The absence of free or membrane as sociated RNP granules in the cytoplasmic droplet is consistent with its lack of baso- philia We are inclined however to ascribe the PAS and acid phosphatase re activity of the droplet to the larger mem branous inclusions just described for these alone are not shared by the confluent PAS and phosphatase negative cytoplasm of the middle piece Furthermore their periph eral location coincides with the previously described distribution of the PAS and acid phosphatase reactions within the droplet

*The annulus*

The annulus which we are unable to identify in our microscopic preparations is conspicuous enough in epididymal sperm when viewed under the electron micro- scope and has been well described by Fawcett and Ito (65) Whether it persists in mature intra uterine sperm we are un able to state with certainty for unfortu nately clear sections at the precise level and plane necessary to determine this point were not available The densities identified in the indicated cross section of the tail in figures 2 and 16 we believe to be portions of the annulus but there is suf

ficient lack of definition here tht we can not be certain The annulus is well shown however in the longitudinal section of an epididymal spermatozoon in figure 10 where it has been bissected at two points on opposite sides of the tail In section it appears accuminate and triangular and reveals a dense homogeneous texture which contrasts sharply with the closely ap posed but less dense terminal members of the mitochondrial sheath According to Fawcett (personal communication) the annulus has originally two distinct com ponents a larger less dense part and a smaller denser part In the bat only the smaller component persists in epididymal sperm

*The head*

Earlier observations on bat spermatozoa emphasized the fine structure of the flagel lum (Fawcett 58 62 Telkka Fawcett and Christensen 61) but Tawcett and Ito (65) have now provided a detailed de scription of the head as well in epididymal sperm The organization of the head in uterine sperm does not differ in any essen tial feature from that of epididymal sperm as described by Tawcett and Ito a point which becomes readily apparent if our figures of intra uterine sperm are com pared with those of Fawcett and Ito We shall therefore mention only features not emphasized by these authors or which ex tend their observations relative to particu lar organizational details

The compressed nucleus has a remark able symmetry the radius of curvature of its upper and lower surfaces being ap proximately equal throughout its length (fig 1) This contrasts with the situation in the rabbit sperm head (cf Bedford 64) which that of the bat otherwise re sembles in many respects An unusual feature of bat sperm noted by us and also by Tawcett and Ito is the extension of the

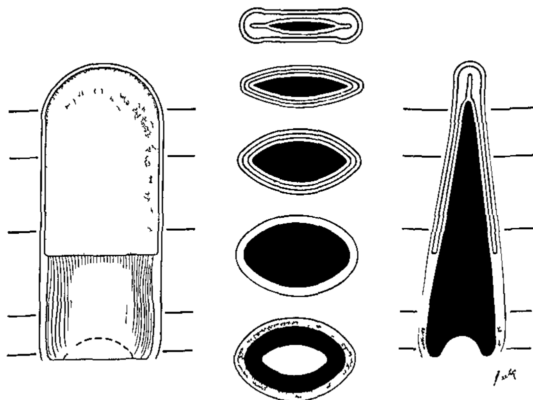


Fig 1 Semischematic reconstruction of the sperm head in *Myotis* based on microscopic and electron microscopic observations The head is depicted at left in three dimensions as viewed in flat aspect far right is shown in longitudinal section a profile view cut at right angles to the plane of flattening Successive transections at levels represented by the hori zontal lines are depicted at center Nucleus black acrosomal cap grey post nuclear sheath stippled cytoplasm white The outer line represents the plasmalemma

uppermost pair of mitochondria of the mitochondrial sheath of the middle piece deep into the basal depression (implantation fossa) of the nucleus The nuclear and mitochondrial outer membranes are in places virtually in contact within this fossa (figs 12 14 17 19) This close juxtaposition of uppermost mitochondria and nucleus has not been indicated in reference to the sperm of other mammals The apparent constriction of the neck region observed by light and phase contrast microscopy is not evident in electron micrographs In the former it is presumably an optical illusion created by a lack of density and chromophobia of the neck cytoplasm and its inclusions

We can find little to add to Fawcett and Ito s account of the organization and relations of the acrosomal cap for they appear to be essentially the same in intrauterine sperm The form and relations of the cap in uterine sperm are shown semischematically in figure 1 and in various section views in the electron micrographs (figs 15–18 incl ) For details of its structure consult Fawcett and Ito ( 65) We have noted in contrast to Fawcett and Ito that the inner face of the acrosomal cap is separated from the outer nuclear membrane by a narrow but relatively uniform zone of low density which in our preparations usually contains a finely granular or amorphous material Anteriorly projecting for a distance into the terminal thickening of the acrosomal cap in the midline is a slit like extension of the subacrosomal zone of lower density just mentioned and it sometimes contains but more often does not a continuation of the amorphous material previously mentioned as filling in between the apposed acrosomal and nuclear membranes at lower levels (fig 17) This area has been termed the "subacrosomal space" by Fawcett and Ito ( 65) because it appeared to be empty in epididymal sperm The entity in question resembles and corresponds to the "apical cone or "apical body described by Hadek ( 63) and Bedford ( 64) in rabbit sperm On the other hand as noted by Fawcett and Ito ( 65) as well as ourselves the discrete triangular density which constitutes the apical body proper of rabbit sperm is absent in bat sperm The designation "cone" is inappropriate in any event for what appears to be conical or slit like in section view has in reality the three-dimensional aspect of a semicircular lamina or ridge disposed parallel to the normal (flat) plane of the head This "lamina" is tallest nearer the center of its arc but tapers in the lateral directions and disappears near the edge of the anterior curved segment of the nucleus "Apical lamina" would be a more useful designation for this structure than "cone " for it is conical only in section profile

Austin and Bishop ( 58) contend in reference to other mammalian sperm that this laminate entity or region is in fact comprised of modified nuclear membrane Our micrographs of bat sperm provide no support for this notion for the apical nuclear membrane immediately beneath the area in question is discrete and unmodified Nor are we persuaded that the term "subacrosomal space" proposed by Fawcett and Ito ( 65) and Fawcett (in press) is altogether appropriate for we have sometimes detected amorphous material within it as already mentioned We believe the latter material when present to be cytoplasmic matrix for it appears continuous with the amorphous material separating the acrosomal and nuclear membranes posteriorly and which is in turn continuous with the thin investing layer of cytoplasm surrounding the basal third of the nucleus below the termination of the acrosomal cap The discrete dense "apical body" of rabbit sperm (Hadek 63 Bedford 64) is presumably homologous with the "perforatorium" of murine and cricetine rodents It should again be emphasized that these discrete structures have not been observed in bat sperm by us or by Fawcett and Ito ( 65)

The organization of the marginal region of the sperm head below the posterior end of the acrosomal cap that is the area of the "post nuclear cap" of earlier cytologists has been described in some detail by Fawcett and Ito ( 65) in epididymal sperm The organization of this region in intrauterine sperm is generally similar and is depicted in figures 14 15 17 and 18 but differences between earlier observations and ours must be noted Fawcett and Ito

the axial complex These tend to be disposed in tandem extending radially to the circle defined by the columns of the connecting piece Some of these granulae are homogeneously dense but others appear to have paler centers It is our belief that these granulae are in fact transections of elements of the axial filament complex near their point of origin from the columns of the connecting piece This view is strengthened by the details indicated by arrows in figures 19 and 20 Here are seen sharply defined fine parallel lines separated by a less dense intermediate layer the whole having a width corresponding to that of an individual axial filament These structures are identified with the granulae previously noted and represent longitudinal or tangential sections of the axial filaments Their peripheral location close association with the columns of the connecting piece and their lack of continuity with the proximal centriole above underscore our belief that the axial filaments originate from the columns of the connecting piece

Fawcett and Ito ( 65) have already drawn attention to the aberrant form of the uppermost pair of mitochondria which lie in the neck and extend deep into the implantation fossa of the nucleus (figs 12 14 17) We can confirm all of their statements relative to the form and relations of these mitochondria including the presence of peculiar pleats or folds which extend between the segmented columns of the connecting piece into the central area occupied at lower levels by the axial filament complex (figs 15 16 19 20) There would seem little doubt that this constant and unique configuration of the neck mitochondria has some special functional significance perhaps in relation to the initiation of flagellar movements as Fawcett and Ito suggest A further peculiarity of this pair of mitochondria also recognized by Fawcett and Ito but not given by them the emphasis it may well deserve concerns their spatial orientation relative to the lower mitochondria of the middle piece As these authors have emphasized the mitochondria of the middle piece in the sperm of *Myotis lucifugus* have a more regular arrangement than observed in most mammalian sperm They are tightly packed and arranged serially in pairs the members of each pair lying at approximately the same level on opposite sides of the middle piece As seen in transection each mitochondrion has a plump crescentic shape and the truncated ends of each member of a pair meet in intimate contact along a plane which roughly corresponds to the dorso ventral axis of the flattened head (i e the x y" axis of Telkka et al 61 Fawcett 62 and the x x axis of Fawcett and Ito 65) By contrast while the pair of neck mitochondria are less regularly crescentic and their ends seldom of ever actually meet they are oriented at right angles to those in the middle piece Were their ends to meet it would be in a plane which coincides with the horizontal plane of the head (i e the y y axis of Fawcett 62 and Fawcett and Ito 65) These relationships may be visualized in figures 12 14 and 17 As to the significance of this axial rotation of the modified neck mitochondria we have at present no suggestion to offer

*The middle piece*

We have little to add here to the observations of Fawcett and Ito ( 65) except to clarify the level and sequence of termination of the nine outer dense fibers Their size rapidly diminishes in the lower half of the middle piece and as observed in the sperm of man and monkey but unlike the sperm of rat mouse and guinea pig they usually extend only a relatively short distance into the principal piece The order of their disappearance with the exception of numbers 3 and 8 is in direct relation to their initial thickness Fibers 3 and 8 which are intermediate in size disappear first (see below) They are followed by fibers 2 4 and 7 The four thickest fibers 1 5 6 and 9 disappear last with nine usually dropping out a little before the others (cf figs 2 15 16 19)

*The principal piece*

Our original observations on the fine structure of the fibrous sheath will not be presented for they add nothing to the detailed account of Fawcett and Ito ( 65) We shall comment on only one aspect of this region in which our observations differ somewhat from those of previous

vorkers and this involves a possible rela ionship between outer dense fibrils 3 and 3 and the longitudinal columns (raphes) formed on opposite sides of the principal piece by junction of the arcuate ribs of the fibrous sheath Previous authors have made no definite reference to possible con nections between outer dense fibers 3 and 8 and the elements of the fibrous sheath despite the fact that these fibers lie on opposite sides of the axial filament com plex approximately on the X Y axis and hence immediately beneath the longitu dinal columns of the sheath on each side of the tail Connections between dense outer fibers which are possibly contractile and the fibrous sheath which may have supportive or elastic properties (Fawcett 62) could if they exist have an important bearing on the dynamics of tail movement Figure 9 section no 1 is a tangential sec tion through the junction between middle piece and principal piece in an epididymal spermatozoon To the left it clearly por trays the ribs of the fibrous sheath joining the slender longitudinal column To the right at the junction with the middle piece the column seems to continue on beyond the fibrous sheath into the middle piece where clearly it can no longer be column substance it must be one of the coarse filaments 3 or 8 The picture by itself suggests that connections may exist between the elements of the sheath and fibrils 3 and 8 but the possibility exists that the coarse fiber passes beneath the raphe in line with it only creating an illu sion of continuity between the two How ever relations as viewed in cross-sections of the principal piece are more suggestive and it may be significant that fibers 3 and 8 while not the smallest are the first to disappear posteriorly Within the mid section of the middle piece fibers 3 and 8 are smaller in cross sectional diameter than the largest fibers 1 5 6 and 9 but noticeably larger than 2 4 and 7 (fig 15) Their outline here is ovate and their long axes are directed radially the larger end facing outward Normally too they are displaced outward somewhat and this be comes accentuated in the lower middle piece and upper principal piece Within the latter they often appear to contact or be fused with the inner edge of the thin longitudinal columns of the fibrous sheath This is especially apparent in distorted sections where the various elements may be somewhat displaced here the two dense fibers usually cling to the fibrous columns rather than remaining proximate to axial filaments 3 and 8 In some cases they even appeared to be included wholly within the inner edge of the fibrous sheath These appearances all suggest that there are areas of fusion between outer dense fibers 3 and 8 and the inner ends of the respec tive longitudinal columns of the fibrous sheath Distally the broad inner ends characteristic of the longitudinal columns at higher levels are replaced by inwardly directed narrow projections of column sub-stance whose ends may touch or nearly touch subfibrils 3 and 8 respectively (figs 2 16 19)

We tentatively suggest that in *Myotis* spermatozoa at least outer dense fibers 3 and 8 gradually blend into the longitu dinal columns of the fibrous sheath rather than ending freely without such attach ment and that this may explain their ap-parent earlier disappearance posteriorly in the principal piece despite their inter mediate size The direction of flexion of the tail in swimming movements is pri marily perpendicular to the plane which bisects outer dense fibers 3 and 8 (cf Fawcett 62) If the latter are contractile they could conceivably exert tension on the fibrous columns in the direction of their longitudinal axis thereby stiffening or polarizing the resistance of the sheath in the dorso ventral (x y) axis to the con traction waves that pass along the other elements of the axial filament complex this might provide in the words of Faw cett (62) " the necessary couple for the transverse bending movements of the tail"

## DISCUSSION

For the most part our observations of sperm ultrastructure in *Myotis lucifugus* have paralleled those of Fawcett and Ito (65) We have confirmed many of their findings and concur in most of their interpretations Accordingly we have re stricted the presentation of our own ob-servations to those necessary to help clarify difficult points or which comple

ment and extend the findings of these authors The Fawcett and Ito study was devoted wholly to epididymal sperm The emphasis in our study of ultrastructure has been upon stored intra uterine sperm since we were especially interested in discovering adaptations which might help explain the remarkable longevity of bat sperm in the female reproductive tract However as far as cytology and ultrastructure are concerned we have found no special modifications which might be related to sperm survival Uterine sperm in hibernating *Myotis lucifugus* resemble in all essentials unejaculated sperm from the epididymis The cytoplasmic droplet characteristic of epididymal sperm is lost at ejaculation but this is usual among mammals Whether the loss by uterine sperm of the redundant nuclear membrane material ( membranous scrolls of Fawcett and Ito 65) so characteristic of epididymal sperm has any special significance for sperm survival we cannot definitely say but we doubt it It is conceivable that the disappearance of the redundant nuclear membrane is in some manner related to the process of sperm capacitation within the uterus but on the other hand the reversibility of the capacitation phenomenon under appropriate experimental conditions (Chang 57 Bedford and Chang 62) makes this too seem unlikely We are forced to conclude that the mechanism of sperm survival in the female tract most probably resides either in special physiological characteristics of bat sperm which have very subtle or no morphological expression or alternatively in physiological adaptations of the uterus itself Our observations concerning the latter possibility will be presented in a later communication

In the process of spermatogenesis significant amounts of cytoplasm are sequestered into the lumen of the seminiferous tubules These residual bodies (Regaud 01) have recently been studied with both the light microscope (Firlit and Davis 65) and with the electron microscope (Deitert personal communication in press) The residual bodies possess PAS positive granules Deitert has also described a large number of lysosomes of varying structure within the cytoplasmic remnants The cytoplasmic droplet present in the epididymal sperm of this study but not in the intra uterine sperm is similar in many respects to the residual body Our observations indicate that the vesicular bodies in the droplet are PAS positive and in many instances probably also contain acid phosphatase Both of these reactions are characteristic of lysosomes (Novikoff 60) It appears then that the cytoplasmic droplet is the last remnant of unsequestered cytoplasm and is related to the residual body With maturation of the epididymal sperm this element like the residual body is also lost

We wish finally to call attention to an odd discrepancy between our microscopic and ultrastructural observations of the middle piece which may be of great functional importance and which has not been commented upon by previous workers It is obvious in bat sperm that far more mitochondria are identifiable in electron micrographs of the middle piece than are revealed by conventional staining procedures of light microscopy indicating that some mitochondria are stained by the conventional methods whereas others are not Moreover in microscopic preparations stained mitochondria alternate in a more or less regular pattern along the longitudinal axis of the middle piece and the stained ones tend to be staggered on its opposite sides i e stained mitochondria on one side of the middle piece generally lie opposite unstained ones on the other side and so on (fig 4) It is this alternation of stained and unstained mitochondria on each side and their phase differences on opposite sides of the middle piece which create the illusion of a continuous mitochondrial spiral for at the low resolving power of the light microscope discontinuities between individual mitochondria are easily overlooked Moreover the frequency with which the mitochondrial component of the middle piece has been described as a continuous spiral or helix suggests that comparable alternations of staining may characterize other mammalian sperm and lessens the likelihood that it has a merely random or fortuitous basis

While this line of reasoning may explain how a misconception of middle piece organization might have arisen the phenomena itself could conceivably have

portant physiological significance in relation to initiation and coordination of sperm tail movements Why do some mitochondria stain whereas others do not and why does their stainability alternate more or less regularly on one side and in staggered on opposite sides of the middle piece? The bases of mitochondrial staining by conventional methods are little understood but they are presumably related to the chemical constitution of the organelles Reversible or cyclic physiological activities within the mitochondria might conceivably alter their chemical makeup in such a way as to affect their stainability It would be interesting to know whether certain mitochondria are permanently stainable and others non stainable or whether they are able to turn "on and off" in time relative to their staining affinity This is not determinable in microscopic preparations of killed sperm but a rarely observed condition in the neck region of sperm in epididymal smears may provide a clue In the preponderant majority of such sperm when stained by conventional methods for mitochondria the neck segment appears achromatic and transparent the uppermost gyre of the stained mitochondrial sheath lying some distance below the head We have observed in such preparations an occasional spermatozoon in which the uppermost stained gyre extends through the neck and abuts against the nucleus (fig 4) In these occasional sperm the aberrant pair of mitochondria in the neck region evidently stain whereas in the vast majority of spermatozoa they do not It is conceivable that these differences in the staining affinity of the peculiar mitochondria of the neck in different spermatozoa reflect a time lapse difference in their activity If this can occur in the neck mitochondria there is no reason to suppose that it cannot among the mitochondria at lower levels in the middle piece Interpreted in this light the *apparent* helical arrangement of stained and unstained mitochondria takes on additional significance for it is the appearance we would expect if the organelles fired in cyclic or pulsed fashion and in sequence along the middle piece Experiments to test this possibility are currently in progress

If this speculation has any validity then important questions (presumably answerable only in the domain of molecular biology) are raised concerning (1) the initiation and control of cyclic mitochondrial activities in the middle piece and (2) the dynamic relation between the pulsed sequential mitochondrial activity and the functional coordination of the individual contractile elements of the axial filament complex responsible for the spatially oriented movements of the tail Successful demonstration that the mitochondria of the sperm middle piece are capable of coordinated cyclic functional sequences might also have more general significance in relation to the nature of mitochondrial activities in other types of cells as well as to the factors which determine their stainability by conventional cytological procedures

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## PLATE 1

### EXPLANATION OF FIGURES

3 Epididymal sperm suspension PAS hematoxylin and light green Only the cytoplasmic droplets are PAS positive all shown are located at the distal end of the middle piece × 1 500

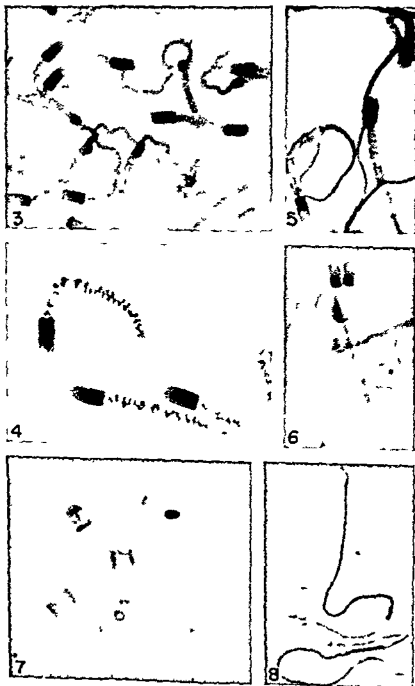
4 Epididymal sperm suspension Altmann aniline acid fuchsin methyl green Cytoplasmic droplets are absent in these sperm Note the prominence of the middle piece within it the axial filament complex appears as a single strand and the apparent helical winding of mitochondria is clearly visible The unstained translucent acrosomal cap renders the anterior end of the nucleus indistinct × 2 400

5 Epididymal sperm suspension hematoxylin eosin and aniline blue Aniline blue stains selectively the fibrous sheath of the principal piece so the junction of the latter with the middle piece is sharply defined Cytoplasmic droplets not present × 1 500

6 Epididymal sperm suspension PAS and hematoxylin Abnormal spermatozoon with two heads and partial division of the middle piece The cytoplasmic droplet which is PAS positive is not terminalized on the middle piece as in most normal sperm in this preparation × 1 500

7 Epididymal sperm suspension Bodian protargol method Only the post nuclear sheath is stained by the Bodian procedure The stained area may not extend all the way forward to the posterior margin of the acrosomal cap × 4 000

8 Epididymal sperm suspension Holmes silver stain The procedure is highly specific for the fibrous sheath of the principal piece and the inner axial filaments Note the peculiar spatulate density at the terminal end of the principal piece characteristic of all sperm stained by the Holmes procedure Its structural basis and significance is unknown The post nuclear sheath region is faintly indicated but the remainder of the head is unstained × 1 500


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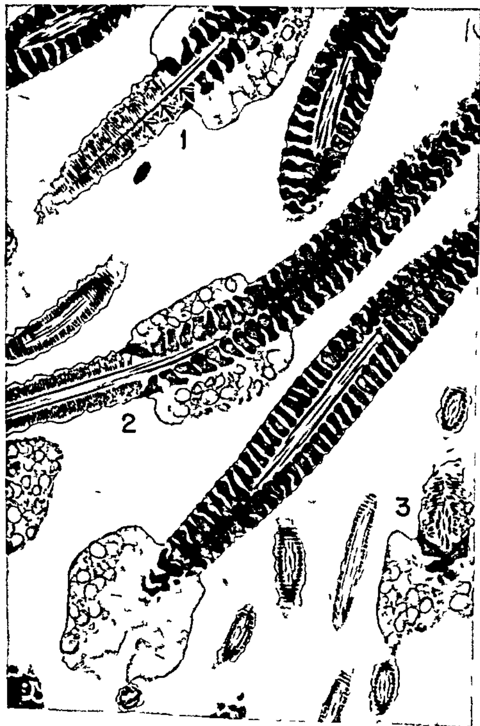
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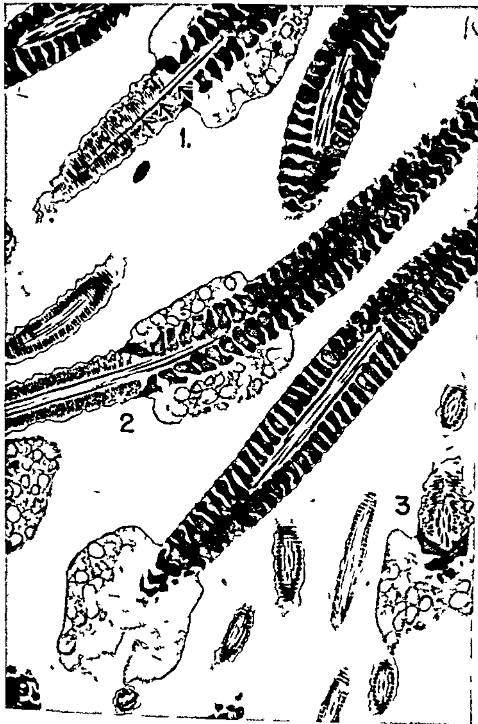
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2
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## PLATE 2

### EXPLANATION OF FIGURE

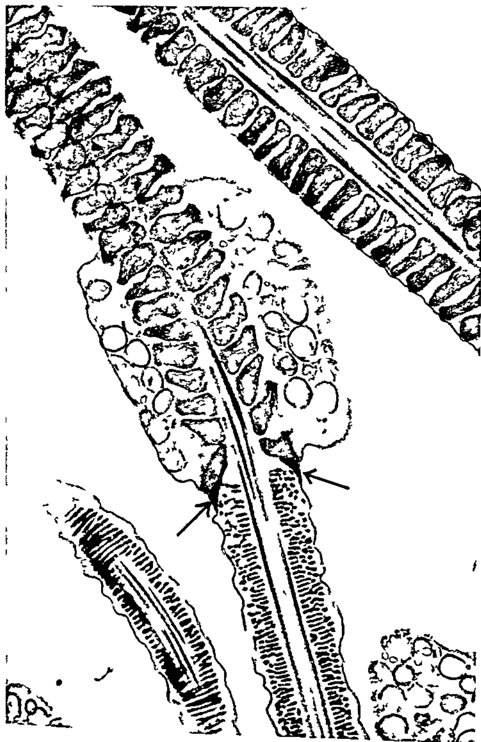
9 Epididymal spermatozoa showing structure and relations of middle piece principal piece and cytoplasmic droplets Three junctions between middle and principal pieces are depicted (1 2 3) note in each the intimate relation between lower mitochondrial pair and annulus and the firm adherence of the cell membrane to the outer face of the annulus In the upper section (1) arrows indicate the apparent confluence described in the text between one of the fibrous longitudinal columns of the principal piece and outer coarse fibril 3 or 8 of the middle piece


1.
2
3

## PLATE 3

EXPLANATION OF FIGURE

10 Enlargement of section 2 from preceding figure showing detail of cytoplasmic droplet and annulus (arrows) The peripheral distribution and organization of the membranous semi vesicles in the droplet cytoplasm is visible here as well as in the preceding and following figures The section plane parallels the flat horizontal plane of the head i e is perpendicular to the X–Y axis of the tail

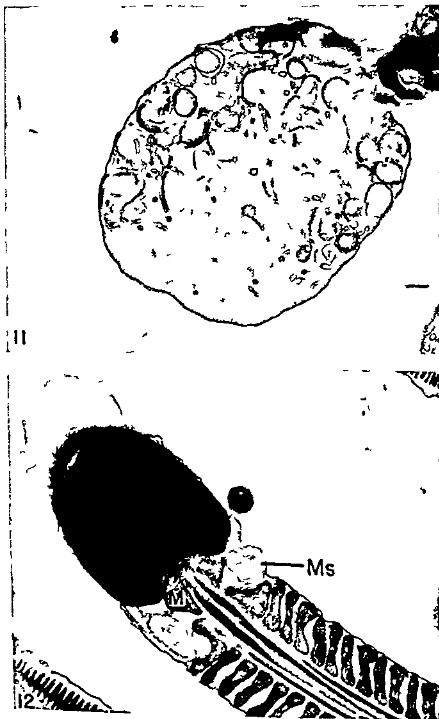


## PLATE 4

### EXPLANATION OF FIGURES

11 Cytoplasmic droplet enlarged showing membranous semi vesicles and tubules smaller vesicles and the tendency for these structures to occupy a peripheral location in the droplet Note absence of RNP particles and lipid droplets The membranous bodies are believed responsible for the PAS and acid phosphatase reactivity of the droplet (see text)

12 Tangential section through base of head neck and upper middle piece of an epididymal spermatozoon The section is off center in relation to the axial complex of the middle piece and is perpendicular to the X–Y axis Note that the uppermost mitochondrion appears unpaired in this plane of section crossing the midline perpendicular to the X–Y axis and skewed 90 in relation to the orientation of the lower pairs in the middle piece Note also its intimate association with the nucleus within the implantation fossa The section is not central enough to include the upper part of the connecting piece but two of its segmented columns are shown just below their junction *M* mitochondrion *Ms* membranous scroll (nuclear membrane)


11
Ms
M
12

## PLATE 5

### EXPLANATION OF FIGURES

13 Epididymal spermatozoa stained by a modification of the Gomori lead sulfide method for acid phosphatase utilizing Na B glycerophosphate as substrate incubation time 30 minutes Note the reactive bead at the junction between middle piece and principal piece representing the cytoplasmic droplet The apparent staining of the fibrous sheath is less constant and is presumed to be an adsorption artifact The same is probably true in respect to the bands on the head but the staining of the unidentified components at the top and base of the neck is consistent and is presumed to reflect enzymic activity No staining occurred in control sections treated with NaFl

14 Intra uterine spermatozoon whose head is embedded in an epithelial cell The plane of section corresponds to the X–Y axis of the axial complex of the tail i e perpendicular to the plane of flattening of the head but it is tangential and off center The plane is at right angles to that of figure 12 with which it should be compared The dense structure (*) in the nuclear depression is apparently part of the connecting piece The skewed orientation of the uppermost mitochondrial pair (only one member shown) is indicated The arrows show location of post nuclear sheath material M mitochondria

13

M

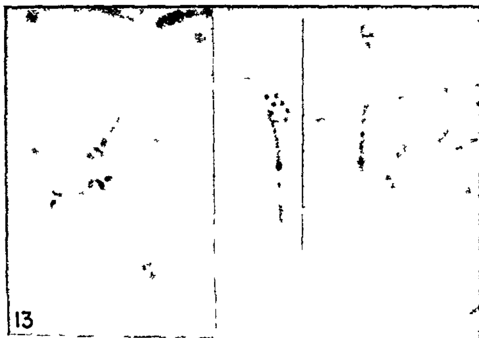
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M

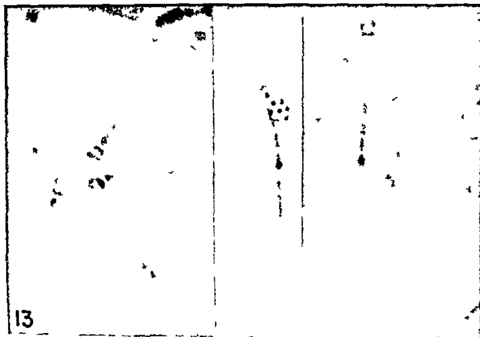
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13

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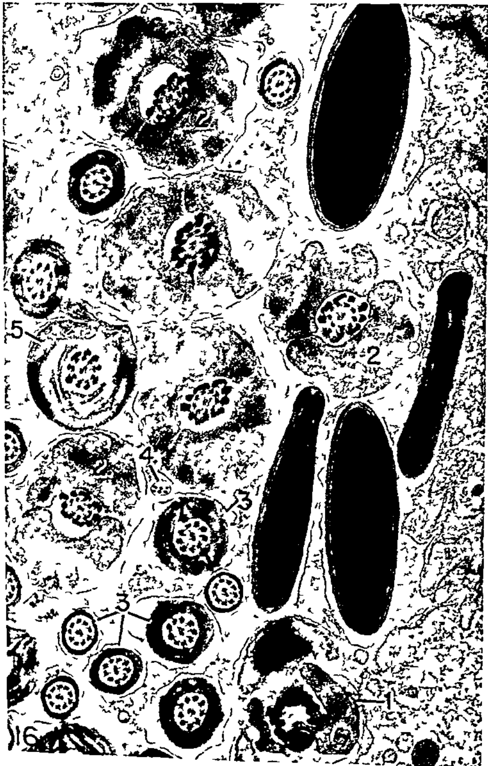
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x
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3
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15

## PLATE 6

### EXPLANATION OF FIGURE

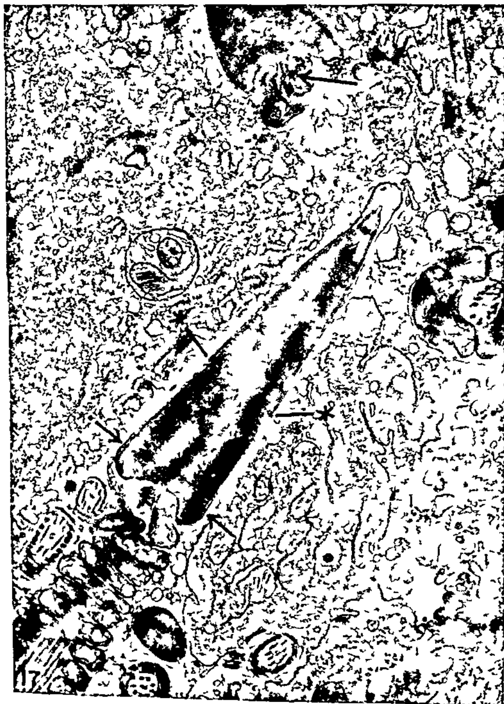
15 Sections at various levels of spermatozoa lying within lumen of uterus (1) transection of head through flattened nuclear apex the acrosomal cap is complete and the lateral ends of its terminal bead (cf fig 1) are shown (2) transection of head at posterior margin of acrosomal cap which is deficient between arrows (3) transection of head posterior to acrosomal cap (4) transverse section through neck showing lower part of connecting piece (segmented columns) subfibrils near their point of origin and mitochondrial pleats (5) sections through lower neck or upper middle piece showing uniform size of the nine outer dense fibers at this level (6) typical transverse sections of middle piece showing larger size of outer dense fibers 1 9 5 and 6 and intermediate size of 3 and 8 the X–Y axis (of Fawcett 62) is indicated on one of the sections (7) section through upper part of principal piece showing association of outer dense fibers 3 and 8 with inner ends of fibrous longitudinal columns (8) section of end piece


2
2
5
2
4
3
3
1
16

## PLATE 7

### EXPLANATION OF FIGURE

16 Sections of intra uterine (luminal) sperm cut at various levels and planes mostly transverse For detailed description see text (1) transections through neck (2) transections of middle piece (3) sections of principal piece at various levels showing variations in thickness of fibrous sheath and alignment of longitudinal columns on X–Y axis of tail (4) section near tip of end piece showing reduction of subfibrils to a ratio of 7+1 (5) transection at junction between middle piece and principal piece elements of the fibrous sheath are visible adjacent to the outer dense fibers and outside them portions of the lowermost pair of mitochondria of the middle piece the densities lying left and right are interpreted as portions of the annulus ( End ring ring centriole )


17

## PLATE 8

EXPLANATION OF FIGURE

17 Intra uterine spermatozoa embedded in epithelial cell In the large central section the head is cut slightly tangentially and in the plane of the X–Y axis of the tail i e at right angles to the plane of flat tening of the head Note especially relations in the neck the acrosomal cap which is shown in its full extent (*= its caudal margin) and the apical lamina The arrow indicates in the upper sperma tozoan a portion of a proximal centriole The lower arrows indicate the basal thickenings of the post nuclear sheath presumed responsible for the argyrophilia of this region


18

## PLATE 9

### EXPLANATION OF FIGURE

18 Tangential longi section of intra uterine spermatozoa lying within the lumen of a gland the section planes are approximately per pendicular to the plane of flattening of the head i e they parallel the X–Y axis of the tail Arrows indicate the basal post nuclear sheath thickenings The full extent of the acrosomal cap is visible in the larger head section


2
MI
B
2
1
3
20

## PLATE 10

### EXPLANATION OF FIGURES

19–20 Transections of sperm within the uterine lumen showing organization of tail segments at various levels (1) neck region (2) upper middle piece (3) upper principal piece (4) more distal levels of principal piece In the neck sections (1) are seen the segmented columns of the connecting piece (B) and the mitochondrial laminae which project between them (see text) Note in the middle piece the larger size of outer coarse fibrils 1 5 6 and 9 the intermediate size of 3 and 8 and the smaller size of 2 4 and 7 The axial planes and other organizational details previously described by others are here confirmed The two spermatozoa represented by sections through the distal portion of the middle piece (4) were facing in opposite directions the observer is looking toward the head in the upper one and toward the tail tip in the lower one The arrows indicate tangential or longi sections of the inner 9+2 array of sub fibrils of the axial filament complex near their point of origin in the connecting piece *M* mitochondria *N* nucleus

# The Effect of Relaxin on the Endometrium of Monkeys

## HISTOLOGICAL AND HISTOCHEMICAL STUDIES [1]

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*ABSTRACT* Castrated or juvenile monkeys were given estrogen progesterone and relaxin in various doses and combinations Relaxin induced (1) a dilatation of the superficial endometrial blood vessels and proliferation of their endothelial cells this effect may be regarded as due to relaxin alone (2) an intensified differentiation of the endometrial stroma cells into predecidual cells and granulocytes dependent on estrogen priming and the simultaneous injection of progesterone (3) a periarteriolar mantle like accumulation of granulocytes in the basal endometrium dependent on the above pretreatment (4) a degranulation of the granulocytes and hypersegmentation of their nuclei following prolonged administration of relaxin with estrogen and progesterone Morphologically and histochemically the granulocytes of the monkey are almost identical with those of the human uterus and with the granular cells in the decidua and mesometrial gland of the pregnant rat Immunohistologically relaxin has been demonstrated in the granulocytes of man and rat The changes in the arterioles brought about by exogenous relaxin occur under physiological conditions only with the increased formation of endogenous relaxin during early pregnancy They have been described in the immediate vicinity of ovum implantation in man monkey and rat Their function possibly lies in the preparation of blood spaces for nutrition of the young embryo

Hisaw and Hisaw (64) studied the effects of relaxin in the female monkey stressing its influence on the thickness of the endometrium and myometrium and on the cervix and symphysis pubis They concluded that relaxin tended to intensify the synergistic action between estrogen and progesterone on endometrial growth Since granulocytic cells occur in the endometrium of the monkey (Cleveland 41 Bartelmez et al 51 Hellweg 59) and resemble the endometrial granulocytes of the human female and since relaxin has been demonstrated immunohistologically in the endometrial granulocytes of the human uterus (Dallenbach and Dallenbach Hellweg 64) a histological and histochemical study of the endometria of these monkeys treated with relaxin seemed to be of great interest It appeared important therefore to determine if exogenously administered relaxin in certain dosages and in combination with estrogen and progesterone would have an effect on the number and distribution of the granulocytes and on the degree of granulation Close attention was also given to the presence of other morphologically recognizable effects which might eventually be ascribed to relaxin in addition to the increased endometrial growth recognized by Hisaw and Hisaw

### MATERIAL AND METHODS

The endometria of 64 monkeys (*Macaca mulatta*) were examined histologically and histochemically using essentially the same uteri which were studied by Hisaw and Hisaw (64) Since the treatments of the castrated animals were presented in detail in their paper they are not included here Ten juvenile and non-castrated animals (nos 286–291 294–297) have been added to the treatment group Controls consisted of 12 uteri of non-castrated untreated monkeys (ten obtained during the proliferative phase and two from the third month of pregnancy) these specimens were also used in a previous publication (Hellweg 59)

Paraffin sections of these uteri were stained with the following *histological techniques* hematoxylin-eosin phloxine

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but without the previous estrogen adminis tration shows predecidual change and many granulocytes The number of granu locytes which develop only in the presence of progesterone is closely proportional to the amount of progesterone administered On the other hand under the influence of progesterone alone there is only little growth and consequently neither prede cidual cells nor granulocytes develop (no 226) With progesterone given over prolonged periods (56 days no 231 234) a degranulation of the numerically unal tered granulocytes is evident If relaxin is added to estrogen and progesterone the differentiation of the stromal cells into the pre-decidual type and into granulocytes is still further enhanced The pre decidual cells appear somewhat larger and the granulocytes seem to increase moderately in number (from +++ to ++++) the shape of the granulocytes and the granulation of their cytoplasm remain un changed (fig 2) Following the adminis tration of only estradiol and relaxin (nos 288 290 292) only a few granulocytes are demonstrable and after estriol alone followed by estriol and relaxin (no 233) still fewer granulocytes are evident than when progesterone is administered simul taneously When only progesterone and relaxin are given in the total (no 253) or in the final treatment (nos 249 and 287) the granulocytes become numerous

When progesterone with or without re laxin is administered over a prolonged period of time (56 days nos 231 234 56 days no 256 with 41 days relaxin) an intense *proliferation and differentiation of the periarterial cells into granulocytes* becomes especially striking in the basal layers of the endometrium Here these granulocytes invest the spiral arteries to produce thick mantles (fig 3) Such peri arterial aggregates of granulocytes are demonstrable under the same hormonal conditions which lead to the differentia tion of predecidual cells and granulocytes The thickness of the mantle of cells about the arteries and arterioles (fig 4) is proportional to the total number of granulocytes in the uterus

The granulocytes in the monkey endometria described here resemble morphologically the granulocytes of the human endometrium hence further description of them is unnecessary The histological and histochemical methods used are com parable to the studies carried out on the granulocytes of human uteri The intra cytoplasmic granules of the granulocytes stain weakly pink with H + E thus they are difficult to recognize With the phloxine tartrazin stain they contrast sharply from the remaining cytoplasmic structures because of their bright red phloxinophilia In addition they are red with the Azan stain Masson s trichrome stain and pentachrome stain With Mal lory s fibrin stain they are violet The granules give a positive tetrazonium re action which becomes negative after ben zoylation The reaction for tryptophan is strongly positive those for histidine ly sine tyrosin and S H groups are also posi tive The isoelectric point as measured with methylene blue on formalin fixed sections is at pH 4 4 The PAS reaction after diastase digestion is weakly positive for some of the granules The same is true for the aldehyde fuchsin stain after oxidation whereas it is negative without oxidation In some of the granules the alcian blue reaction is positive With the alcian blue PAS combination the granules appear blue They fail to give an absolute (alcohol fast) metachromasia but a red color reaction is retained after dehydra tion with acetone They react negatively to Sudan Black B These histochemical results suggest that the granules consist of an acidophilic protein to which pos sibly an acid mucopolysaccharide is more or less loosely bound

(d) In some animals in the the super ficial layers of the endometrial stroma lacunar dilatations of thin walled blood ves sels (altered arterioles and capillaries) are evident which reveal a variably intense *proliferation of their endothelial cells* (fig 5 and 8) Some of the endothelial cells swollen and globular bulge into the lu men (fig 6) others are enlarged to the size and shape of the decidual cells have round chromatin poor nuclei and are often multinucleate (fig 7) They have a homogeneous cytoplasm in which — with the above mentioned histochemical methods — only glycogen and neutral mucopoly saccharides are demonstrable with the

tartrazin after Lendrum Massons tri chrome stain[4] Mallorys trichrome stain[4] Heidenhains Azan stain[4] Movats penta chrome stain[3] Gomoris and Paps retic ulum stain[4] and Mallorys fibrin stain (phosphotungstic acid hematoxylin[3])

The *histochemical reactions* employed were Sudan Black B[2] PAS with and without diastase[2] aldehyde fuchsin with and without oxydation[3] toluidine blue (at pH 4 5 and 7 0)[3] alcian blue (at pH 2 5)[3] alcian blue PAS (according to Manual of Histologic and Special Staining Tech nics AFIP Washington D C 57) coupled tetrazonium reaction with and without benzoylation[2] Adams DMAB (p dimethyl amino benzaldehyde) nitrite method for tryptophan[2] Geyers modification of the Morel Sisky reaction for tyrosine hydroxy naphthaldehyde method for protein bound $NH_2$ groups after Weiss et al [2] reaction for histidine after Bachmann and Seitz reac tion for SH groups after Barrnett and Selig man[2] and determination of the isoelectric point with methylene blue at controlled pH after Pischinger[4]

RESULTS

In table 1 the results of the study of the glands the stromal cells and the blood vessels of the 64 endometria are presented

(a) When viewed under low magnifica tion the variations in thickness of the endometria are very striking Even more striking are the *changing proportional re lationships between the volumes of the glands and of the stroma* In the normal proliferative phase (control cases) and after treatment with only estrogen or progesterone or with these combined in ineffectual doses or with estrogen and relaxin this relationship is 1 1 that is the straight glands extending through the functionalis from the basalis to the sur face occupy about the same amount of space as does the intervening stroma During pregnancy as well as after the com bined administration of estrogen and pro gesterone in physiological doses the gland stroma relationship is altered in favor of the stroma which is greatly thickened and has assumed a predecidual appear ance especially in the more superficial levels of the endometrium (the so called compacta) (fig 1) In contrast the dilated portions of the glands now form the spongiosa and lie adjacent to the basalis The longer progesterone is administered the more the glands regress in the intensely developed almost decidual like stroma (no 231 234) On the other hand the addi tion of relaxin appears to have no effect on this relationship If small doses of progesterone are given relative to a long pretreatment with estrogen the relation ship may be altered in favor of the glands which at times may be found tightly coiled and extending to the surface (no 273)

(b) The *glandular secretion* is essentially dependent on a sufficient dose of proges terone which of course must have beer preceded by an adequate treatment witl estrogen With an additional administra tion of relaxin the progesterone effect or the glands is not changed but hypersecre tion and vacuolization occurs similar t the Arias Stella Phenomenon in women In the absence of progesterone relaxi alone cannot cause the glands to secret (nos 288 290 293)

(c) Since the differentiation of th stroma cells into *predecidual cells* on on hand and into *granulocytes* on the othe run practically parallel to one another th concomitant changes will be considere together It should be noted that th granulocytes in the monkey uterus may b very numerous in the superficial compac zone but occur in the basal portion c the endometrium as well and there i greater numbers than in the huma uterus The differentiation of both typ of cells predecidual cells and granulocyte like the glandular secretion appears onl after distinct combinations of hormones sufficient doses of progesterone and ad quate pretreatment with estrogen or if n estrogen has been given progesterone an relaxin (no 253) If the daily doses c progesterone are too small (no 257) c preceded by a prolonged (80 days) e trogen treatment (from a previous e periment) (no 248) neither predecidu cells nor granulocytes develop normall Monkey 274 treated the same as no 24

[2] See Pearse
[3] See Humason
[4] See Romeis

but without the previous estrogen adminis tration shows predecidual change and many granulocytes The number of granu locytes which develop only in the presence of progesterone is closely proportional to the amount of progesterone administered On the other hand under the influence of progesterone alone there is only little growth and consequently neither prede cidual cells nor granulocytes develop (no 226) With progesterone given over prolonged periods (56 days no 231 234) a degranulation of the numerically unal tered granulocytes is evident If relaxin is added to estrogen and progesterone the differentiation of the stromal cells into the pre-decidual type and into granulo cytes is still further enhanced The pre decidual cells appear somewhat larger and the granulocytes seem to increase moder ately in number (from +++ to ++++) the shape of the granulocytes and the granulation of their cytoplasm remain un changed (fig 2) Following the adminis tration of only estradiol and relaxin (nos 288 290 292) only a few granulocytes are demonstrable and after estriol alone followed by estriol and relaxin (no 233) still fewer granulocytes are evident than when progesterone is administered simul taneously When only progesterone and relaxin are given in the total (no 253) or in the final treatment (nos 249 and 287) the granulocytes become numerous

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TABLE 1

*The left half of this table is a condensation of treatments given to each monkey In all animals receiving estradiol this was given first as pretreatment Where the duration of estradiol therapy exceeds that of the other hormones the period of estradiol pretreatment may be calculated by subtracting the days of estriol or progesterone therapy from the days estriol was given Where the duration of estradiol therapy does not exceed that of the other hormones estradiol was injected only as pretreatment and not included in the final treatment (nos 286 287 291 249 289 285 284 271) Only one monkey was given estriol as pretreatment (no 233) Monkey 248 had received estriol in a previous experiment thus the long estriol therapy In monkeys 289 and 291 relaxin therapy was discontinued two days in 296 and 297 four days before the progesterone treatment ended The right half of the table summarizes the histologic changes observed in the endometria They are indicated by the signs*[1]

| Animal no | Hormone treatment (daily dose — days given) | | | | Results | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Estradiol 17β | Estriol | Progesterone | Relaxin | Gland/stroma ratio | Gland secretion | Predecidual change | Granulocytes | Endothelial proliferation |
| | *10 µg* | *mg* | *mg* | *GPU* | | | | | |
| 11 normal controls | — | — | — | — | 1 1 | — | — | (+) | — |
| 2 abortions | — | — | — | — | 1 4 | +++ | +++ | +++ | ++ |
| 270 | 20d | — | — | — | 1 1 | — | — | — | — |
| 225 | — | 0 5–35d | — | — | 1 1 | — | — | — | — |
| 227 | — | 1–30d | — | — | 1 1 | — | — | — | — |
| 228 | — | 1–34d | — | — | 1 1 | — | — | — | — |
| 226 | — | — | 2–31d | — | 1 1 | — | — | — | — |
| 263 | 45d | — | 0 5–30d | — | 1 1 | — | — | + | — |
| 245 | 51d | — | 0 5–43d | — | 1 1 | — | — | — | — |
| 267 | 51d | — | 1–35d | — | 1 1 | — | — | — | — |
| 237 | 40d | — | 2–20d | — | 1 1 | ++ | — | ++ | — |
| 251 | 39d | — | 2 5–19d | — | 1 1 | ++ | + | +++ | — |
| 259 | 49d | — | 2–31d | — | 2 1 | +++ | ++ | +++ | — |
| 269 | 59d | 1–42d | 1–42d | — | 1 1 | — | — | + | — |
| 238 | 53d | 1–43d | 2–43d | — | 1 2 | +++ | ++ | +++ | — |
| 231 234 | 71d | 1–46d | 2–56d | — | 1 8 | ++ | +++ | ++++ | — |
| 293 | — | — | — | 6000–20d | 1 1 | — | — | — | — |
| 288 290 | 44d | — | — | 6000–20d | 1 1 | + | — | + | +++ |
| 292 | 41d | — | — | 6000–20d | 1 1 | + | — | + | + |
| 295 | 15d<br>0 25 mg–20d | — | — | 6000–20d | 1 2 | + | — | ++ | ++ |
| 233 | — | 0 5–31d | — | [illegible] | | | | | |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| [illegible] | [illegible] | | | | | | | | |
| 237 | 5 d | — | 0 5–3?d | 6000–20d | 1 1 | — | — | (+) | + |
| 273 | 50d | — | 1–35d | 6000–20d | 2 1 | ++ | ++ | ++ | (+) |
| 286 287 | 20d | — | 2–35d | 6000–21d | 1 2 | +++ | ++ | ++++ | +++ |
| ?91 | 20d | — | 2–13d<br>2 5–20d | 6000–18d | 1 1 | ++ | + | +++ | + |
| ?98 | 14d | — | 2–10d<br>2 5–20d | 6000–16d | 1 1 | ++ | + | ++++ | + |
| 264 | 50d | — | 2–35d | 6000–20d | 1 2 | +++ | ++ | ++++ | +++ |
| 249 | 22d | — | 2–37d | 6000–22d | 1 2 | +++ | ++ | ++++ | +++ |
| 289 | 20d | — | 2–13d<br>5–20d | 6000–18d | 1 2 | +++ | ++ | ++++ | +++ |
| 297 | 13d | — | 2–10d<br>5–20d | 6000–16d | 1 1 | ++ | + | ++++ | ++ |
| 256 | 73d | — | 2–58d | 6000–41d | 1 3 | +++ | ++ | ++++ | +++ |
| 248 | 55d | 1–80d | 2–40d | 3000–20d | 1 1 | + | — | (+) | — |
| 265 | 57d | 1–40d | 0 5–40d | 4500–20d | 1 1 | — | + | ++ | (+) |
| 262 | 65d | 1–50d | 0 5–50d | 4500–30d | 1 1 | — | + | + | (+) |
| 240 | 65d | 1–50d | 1–50d | 3000–30d | 1 1 | + | + | ++ | (+) |
| 27? | 31d | 0 25–21d | 2–31d | 3000–21d | 1 2 | +++ | ++ | ++++ | — |
| 274 | 67d | 1–41d | 2–41d | 3000–21d | 1 2 | +++ | ++ | ++++ | — |
| 254 | 26d | 0 5–?0d | 2–30d | 6000–20d | 1 2 | +++ | ++ | ++++ | (+) |
| 266 | 54d | 1–20d | 2–34d | 6000–20d | 1 2 | ++ | + | ++++ | — |
| 279 | 61d | 1–35d | 2–35d | 6000–20d | 1 3 | +++ | ++ | ++++ | (+) |
| 236 239 | 55d | 1–40d | 2–40d | 6000–20d | 1 3 | +++ | ++ | ++++ | — |
| 285 | 20d | 0 25–31d | 2–31d | 6000–21d | 1 2 | +++ | ++ | ++++ | + |
| 284 | 20d | 0 5–32d | 2–32d | 6000–22d | 1 4 | +++ | +++ | ++++ | — |
| 271 | 15d | 0 5–36d | 2–36d | 6000–21d | 2 1 | ++ | — | ++ | — |
| 2 6 | 68d | 1–42d | 2–40d<br>4–22d | 6000–22d | 1 2 | +++ | ++ | ++++ | (+) |
| 272 | 51d | 1–36d | 4–36d | 6000–20d | 1 1 | ++ | — | ++ | (+) |

[1] F r the g n locyte — none; (+) v ry occasional cells in the sup rficial end metrium + few scattered cells mainly in the superficial endometrium; ++ num rou gr nulocytes locall d p imarily in the c mpacta; +++ conspi u us g nulocytes in ll layers of ndometrium ++++ very large num ber f gr nulocytes exceedi g th se of the decidual c lls F r th other changes — non ; + light; ++ mode ate +++ extensive Und r endothelial proliferati n (+) indic tes enlargement of the endothelial cells in a few non dilated subepithelial capillaries

PAS and alcian blue PAS reaction Often the cells are stratified in several layers In two monkeys (nos 288 and 290) some of these blood vessels are almost occluded by plump hillock like clusters of the hyperplastic endothelial cells (fig 9 and 10) They are delineated from the surrounding endometrial stromal cells only by the intact but inconspicuous basement membrane Most of these stromal cells next to the basement membrane are well differentiated granulocytes (fig 7) They replace the missing media and adventitia of these vessels Many of these dilated arterioles and capillaries pursue a tortuous course through the endometrial stroma others run parallel to the surface directly beneath the mucosal epithelium (fig 8) On cursory examination it is easy to mistake these vessels as glands With the alcian blue PAS reaction however they are readily differentiated The epithelial cells of the glands contain a secretion staining intensely blue (acid mucopolysaccharides) whereas the proliferating endothelial cells are filled with a finely granular red substance (neutral mucopolysaccharides and glycogen)

These changes of the arterioles as seen in this study are found only in those animals which received relaxin In addition in this series such changes were observed always and only when comparatively little estriol (0 25 mg no 285) or no estriol (nos 249, 253 256 257 264 278, 286–292) was administered The arteriolar response after 0 25 mg estriol with relaxin (6 000 GPU) appears to be decreased, and 0 5–1 0 mg estriol causes complete inhibition of this response in some endometria whereas in others a few non dilated subepithelial capillaries may have enlarged endothelial cells (see table 1) On the other hand the usual daily dose of estradiol (10 μg) or more does not seem to depress the action of relaxin on the blood vessels Furthermore there are no indications of an inhibitory influence on this action of relaxin when progesterone was given alone (no 253) or in combination with estradiol (nos 249 256 257, 264 286 287, 289) Hence these vascular changes appear after relaxin is administered in the absence of estrogen (no 253) or progesterone (nos 288 290 292) Actually the endothelial proliferation appears most pronounced after relaxin is given with only estradiol (nos 288 and 290) whereas the vascular dilatation is best seen after only progesterone and relaxin in the total (no 253) or final treatment (nos 289 278) Prolonged doses of relaxin effect no further increase in these changes (compare no 256 with 264) The

TABLE 2

*Although this represents a simplification of the results of the hormone treatments this table is supposed to provide a survey of the actions of these hormones when given in doses thought to be physiological and not over prolonged periods of time Hence e g the formation of endogenous relaxin following prolonged treatment with estrogen and progesterone with resulting endothelial proliferation is not considered in this table*

| Treatment | Decidual change | Granulocytes | Gland secretion | Endothelial proliferation |
|---|---|---|---|---|
| E | – | – | – | – |
| P | – | – | – | – |
| R | – | – | – | – |
| E+P | + | + | + | – |
| E+R | – | – | – | + (proliferation enhanced) |
| P+R | (+) | + | + | + (dilatation enhanced) |
| E+P+R | + | + | + | + |

E estrogen P progesterone R relaxin

withdrawal of relaxin 2–4 days prior to discontinuing the progesterone (nos 291 296) brings about a decrease in the vascular dilatation and endothelial proliferation as compared to nos 286 and 287 This decrease fails to occur in monkeys 289 and 297 in which relaxin was also withdrawn 2–4 days early but in which the daily dose of progesterone was twice that given to no 291 Although the estrogen (and relaxin) doses were the same in animals 288 290 (juvenile monkeys) and 292 (an adult castrate) the latter had a reduced endothelial proliferation probably because of its castrated state and larger size

(e) In those cases in which the endometrium is especially rich in granulocytes one may observe with silver impregnation techniques a focal dissolution of the reticulum fibers which normally envelop individual stromal cells This is especially evident in monkey 256 which has received relaxin over a long period of time (41 days)

DISCUSSION

In order to evaluate the action of relaxin on the endometrium we must try to separate its activity from the combined effect of estrogen progesterone treatment This is indeed possible from the cases studied (see table 2)

The actions of relaxin on the monkey uterus may be summarized as follows (1) The hormone functions as an intensifier of growth and differentiation of the endometrial stroma especially of the granulocytes but does not initiate these effects With a suitable pretreatment with estrogen and a simultaneous administration of estrogen and progesterone or progesterone alone (nos 249 287) relaxin enhances their effect In the absence of progesterone relaxin remains ineffective on the endometrial stromal cells As suggested by one experiment (no 253) relaxin may be effective on the stromal cells in the absence of estrogen (pretreatment and final treatment) but this possibility requires further testing (2) After prolonged administration and in the presence of adequate amounts of estrogen and progesterone relaxin calls forth an especially rich accumulation of granulocytes about the spiral arteries of the basal endometrium as does a prolonged administration of progesterone without the addition of relaxin (3) Relaxin as well as progesterone when given continuously over long periods of time may eventually lead to degranulation of the granulocytes and to hypersegmentation of their nuclei (4) Relaxin induces an enlargement of the arterioles and capillaries and a proliferation of their endothelial cells in the superficial portions of the endometrium and alone seems responsible for this effect The smallest dose and the shortest time interval needed for the appearance of these changes cannot be determined from these studies although the dose of relaxin given to monkey 278 appears to be quite small (low potency preparation of relaxin) Present results indicate that estriol may be more effective than estradiol in inhibiting the action of relaxin on the endothelium of endometrial blood vessels but this has not been proven on the basis of relative estrogenicity of the two compounds

Of all these effects only the endothelial proliferation in the arterioles and capillaries may be interpreted as due solely to relaxin The occurrence and physiology of these vascular changes in this series of monkeys have been described and interpreted by Hisaw and Hisaw (66) Whereas the granulocytes always occur only with a simultaneous change of the remaining stromal cells into predecidual or decidual cells the endothelial proliferation appears entirely independent of the degree of differentiation of the endometrial stroma and may be seen for example in a non secretory endometrium (nos 288 290 and 292) The enhanced differentiation of the endometrial stroma after relaxin treatment in the presence of estrogen and progesterone is interesting for the following reasons The synergism between estrogen and progesterone which occurs in the presence of endogenous relaxin may be the result of the relaxin action itself hence can be increased by the injection of exogenous relaxin Hisaw and Hisaw (64) showed that in monkeys receiving estrogen and progesterone a formation of endogenous relaxin (0 2 to 0 3 GPU/ml blood serum)

takes place (no 259) its site of formation is to be looked for in the endometrial granulocytes The periarteriolar mantle like accumulations of granulocytic cells like the granulocytes in the remaining decidua are therefore, to be regarded as evidence of an increased formation of relaxin, brought about by progesterone not however as being induced by relaxin The degranulation of the granulocytes probably results from a prolonged excessive action of exogenous or endogenous relaxin that is formed following progesterone therapy over long periods The mechanism of this degranulation remains unclear The decrease in endothelial proliferation after relaxin withdrawal (nos 291 296) did not occur when the daily dose of progesterone was 5 mg (nos 289 297) It is presumed that this dosage of progesterone enhanced the formation of endogenous relaxin in the granulocytes This endogenous relaxin most likely replaced the exogenous relaxin withdrawn

An increase of the *granulocytes* in the endometrium of the monkey after treatment with estrogen and progesterone was also observed by Cleveland (41) This has also been seen in endometrial tissue from castrated women during experimental studies of hormone substitution therapy (Hellweg et al 60) Morphologically the endometrial granulocytes of the human uterus (Hamperl 54 Hellweg 54) and those of the monkey are virtually identical Furthermore both resemble the frequently described granular cells of the meso metrial decidua and the metrial gland of the pregnant rat Histochemically only slight differences exist between the granulocytes of these three species These differences apparently are limited to a prosthetic group bound more or less firmly to the acidophilic protein In monkeys this prosthetic group apparently consists of acid mucopolysaccharides corresponding consequently to that of the similar appearing granules of the rat (Wislocki et al 57) In human granulocytes such a prosthetic group is demonstrable only very rarely for the granules of the granulocytes are present almost always as a pure protein (Hellweg 56) Relaxin has been demonstrated immunohistologically in the endometrial granulocytes of the human uterus and in the granular cells of the pregnant rat (Dallenbach and Dallenbach Hellweg 64 Dallenbach Hellweg et al 65) At the present time we are engaged in a study using similar techniques to determine if relaxin is also present in the endometrial granulocytes of the monkey Differences between the granular cells in women monkeys and rats may be related to their localization and can be interpreted as an adaptation required by the varying types of placentation In the rat these granular cells are present chiefly in the myometrium and appear only during pregnancy and pseudopregnancy In the human uterus they are limited to the endometrium in nonpregnant and to the basal plate of the mature placenta in pregnant women In the monkey these cells are so-to speak intermediary being found in the superficial parts of the myometrium as well as in the endometrium in the nonpregnant animal and in the basal plate of the placenta and adjacent myometrial portion during pregnancy A *periarterial accumulation* of specific decidual cells has been noticed in the pregnant monkey by Wislocki and Streeter (38) and by Ramsey (49) in the human decidua by Ercolani (1873) Waldeyer (1890) Paladino (00) Litwak (31) and Brettner (64) and it is well known as constituting a major part of the metrial gland of the pregnant (Weill 19 and many others) and pseudopregnant rat (Selye and McKeown 35 Velardo et al 53 Ellis 57) It has furthermore been observed in the pregnant mouse (Goldmann 12 and many others) rabbit (Minot 1889 and others) golden hamster (Orsini, 54) and in insectivores *Macroscelides proboscideus* (Starck 49) *Nasilio brachyrhynchus* (Gérard 23) *Elephantulus myurus* (van der Horst and Gillman 46) and *Tenrec ecaudatus* (Goetz 37)

The *endothelial proliferation* similar to that brought about by exogenous relaxin has also been observed under normal physiologic conditions namely during early pregnancy which hormonally provides a favorable state for the secretion of endogenous relaxin apparently exceeding that of the menstrual cycle Such proliferative changes of the vascular endo-

thelium were described by Wislocki and Streeter (38) and by Ramsey (49) in the decidua of the pregnant monkey Wis and Streeter called this "cytomor " Because the walls of these en rged vessels have undergone such a with an apparent disappearance the medial and adventitial coats the essels easily may be mistaken for ven They are however continuous with the spiral arterioles as clearly shown by Ramsey (49) We therefore may assume that the structures of these vascular walls have been changed into or replaced by the granulocytes directly adjacent to the proliferated endothelial cells and separated from them only by the basement membrane The numerous vessels beneath the endometrial surface showing endothelial proliferation are most likely proliferated and greatly dilated capillaries Similar changes of the vascular endothelium have been known in the human uterus and have been described often in the basal decidua (Grosser 27 see literature there Ortmann 55 Wilkin 60) In the mesometrial decidua of the pregnant rat such changes of the arterioles were found in the immediate vicinity of the implantation of the ovum (Goldman 12 Szendi 33 Bridgman 48 Bulmer and Dickson 60 Dallenbach Hellweg et al 65) and beginning on the thirteenth day these modified arterioles were seen together with the first major appearance of the granular cells in the metrial gland (Gérard '25 Selye and McKeown 35 Baker 48) Endothelial proliferation has furthermore been seen in the pregnant rabbit (Minot 1889 Maximow 1898 and others) golden hamster (Orsini 54) guinea pig (Pytler and Strasser '25 Grosser '27) mouse (Goldmann 12 and many others) ewe (Bjorkman 65) and in several in sectivores *Macroscelides proboscideus* (Starck 49) *Nasilio brachyrhynchus* (Gérard '23) *Tenrec ecaudatus* (Grosser '28 Goetz '37) *Sorex vulgaris* (Hubrecht 1894) *sorex fumeus* and *Blarina brevicauda* (Wimsatt and Wislocki 47) That these cells are not of trophoblastic origin in spite of their morphologic similarities with syncytial trophoblastic cells is clearly evident in this series of non pregnant monkeys The proliferating endothelial cells of the rat (Szendi 33) and of the monkey (see the above description) as well as those of the human uterus (Ortmann 55) contain polysaccharides or glycogen Two histologic features suggest that these changes of the arterioles are closely related to the action of relaxin They occur physiologically only with the marked accumulation of the granulocytes and only in their immediate vicinity It is indeed interesting that Rossman (40) saw an identical endothelial proliferation in deciduomas of monkeys and Hisaw ('44) in the endometrium of the monkey after menstruation was postponed by prolonging the functional activity of the corpus luteum with chorionic gonadotropin

The function of these dilatations and endothelial proliferations of the arterioles and capillaries remains to be elucidated It seems tempting to assume that in the preparation for implantation they enhance the blood supply and hence nutrition for the embryo This action would be in accord with the concept that relaxin facilitates implantation and would permit the conclusion that relaxin is a true hormone of pregnancy

ACKNOWLEDGMENT

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## PLATE 1

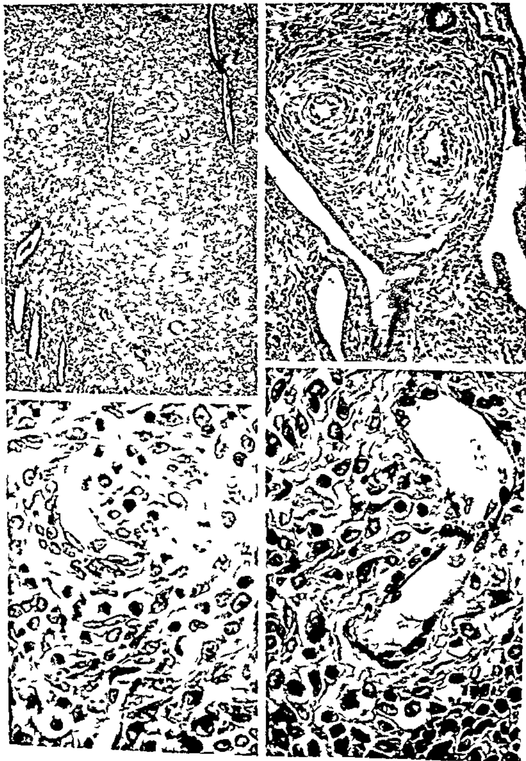
### EXPLANATION OF FIGURES

1 Predecidual appearance of the endometrial stroma with scarcity of glands following administration of estrogen progesterone and relaxin Monkey 239 H and E stain Magnification 56 ×

2 Endometrial granulocytes with paranuclear accumulation of granules and peripheral vacuolization of cytoplasm surrounding a capillary in the compact layer of the endometrium after substitution with estrogen progesterone and relaxin Monkey 285 H and E stain Magnification 560 ×

3 Spiral arterioles in the basal layer of the endometrium surrounded by sheaths of granulocytes following prolonged relaxin administration for 41 days Monkey 256 H and E stain Magnification 138 ×

4 Periarteriolar accumulation of granulocytes in the endometrium of the same monkey as in figure 1 H and E stain Magnification 560 ×



## PLATE 1

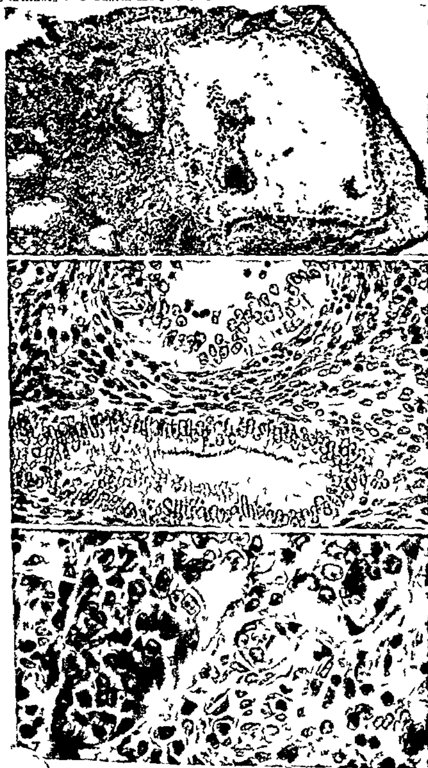
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4 Periarteriolar accumulation of granulocytes in the endometrium of the same monkey as in figure 1 H and E stain Magnification 560 ×



## PLATE 2

### EXPLANATION OF FIGURES

5 Lacunar dilatation and endothelial proliferation of a capillary space underneath the surface epithelium of the endometrium of a monkey treated only with progesterone and relaxin no 253 H and E stain Magnification 90 ×

6 Same monkey as in figure 5 The swollen endothelial cells bulge into the capillary lumen (upper half of the figure) The surrounding stroma and a neighboring gland (lower half) show incomplete progestational change in the absence of estrogen H and E stain Magnification 345 ×

7 Endothelial proliferation with formation of large multinucleated giant cells and partial occlusion of the lumen of an arteriole in the compact layer of the endometrium following administration of estradiol progesterone and relaxin without estriol Next to the basement membrane of the arteriole groups of well differentiated granulocytes can be seen *Monkey 249 H and E stain Magnification 560 ×*



## PLATE 3

### EXPLANATION OF FIGURES

8 Same monkey as in figure 7 Lacunar dilatation and tortuosity of arterioles and capillaries with endothelial proliferation underneath the surface epithelium In the lower middle of the figure a group of undilated spiral arteries At the upper right and lower left straight narrow portions of secreting glands H and E stain Magnification 70 ×

9 Endometrium from Monkey 288 treated only with estradiol and relaxin Proliferating glands and spindle shaped stromal cells in the absence of progestational change In the middle a capillary space occluded by extensive endothelial proliferation Phloxine Tartrazin stain Magnification 175 ×

10 Same monkey as in figure 9 A higher magnification (560 ×) of a capillary with partial occlusion of its lumen by large proliferated endothelial cells Phloxine Tartrazin stain

# The Topographic Anatomy of the Muscles, Nerves, and Arteries of the Bovine Female Perineum

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*ABSTRACT* The perineal muscles and fasciae are described and illustrated and the diaphragma urogenitalis components of m sphincter urethrae and m ischiocavernosus are described for the first time in the cow

The nerves of the pudendal plexus are discussed comparatively with the object of suggesting appropriate names based on their homology to the condition in other animals and man The rami musculares to m. levator ani and m coccygeus may originate from nn sacrales 3 et 4 or n pudendus or n rectalis caudalis The combination of rami musculares with n rectalis caudalis was formerly called by veterinary anatomists n hemorrhoidalis medius a term which should be abandoned because it is not listed in *Nomina anatomica* and because it has also been applied to n splanchnicus pelvinus in the horse N pudendus is large and its rami cutanei supply regio femoris caudalis as well as regio perinealis It also gives rise to n perinealis profundus which supplies the genital muscles The pudendal nerve ends by dividing into ramus mammarius and n dorsalis clitoridis There may be one or two nn rectales caudales They supply m. sphincter ani externus and adjacent parts of m levator ani and the genital muscles

The distribution of arteria urogenitalis (vaginalis) and a pudenda interna are described and illustrated

In spite of the clinical importance of the perineum of the cow no comprehensive study of it has been published A brief description was included in a dissection guide (Habel 49 64) Various authors have described the muscles (Geiger 54 Bassett 61) and the blood vessels (Vollmerhaus 64) but the nerves have been described only in the male (Larson and Kitchell 58)

The purpose of this study was to prepare topographic descriptions and illustrations of the muscles fasciae nerves and arteries The veins were treated briefly as they were encountered in the dissection Problems of comparative nomenclature of these structures required extensive reference to the literature of systematic anatomy in the effort to determine valid homologies among the domestic quadrupeds and man Two essential criteria of veterinary anatomical nomenclature are homonymy of homologous structures in quadrupeds and the closest possible agreement with human nomenclature the latter being modified to eliminate reference to the anatomical position Thus the scope of the study was enlarged to include systematic and comparative considerations and these are taken up in the discussions of individual structures The genital organs and the anal canal were not examined because they have been adequately described in numerous works (Martin and Schauder 38 Schummer and Nickel 60)

## MATERIALS AND METHODS

In addition to the specimens examined in the dissection room where six or seven cows are dissected each year by veterinary students using a laboratory guide that directs a study of the perineum (Habel 49) 22 preparations from 16 cows and six female calves were dissected especially for this investigation Sixteen animals were fixed by arterial injection of 8% formalin and 2% phenol in water usually with the subject in the standing position (Lambert 16) After fixation the arteries were injected with colored starch glue or latex Six preparations of the pelvic viscera were dissected in the fresh state with the aid of a stereoscopic microscope Photographs and drawings were made during the dissections

argued that the udder is not homologous to the scrotum but a similar objection applies to the ventral (anterior) limit of the human perineal region which includes the labia and excludes the scrotum The perineal region was divided by a line connecting the ventromedial processes of the tubera ischiadica into an anal region and a urogenital region

The perineal body lies in the median plane on the intertuberal line In the cow it was formed by the junction of four striated muscles superficially and by a deeper mass of connective tissue and smooth muscle The four striated muscles were sphincter ani externus constrictor vulvae transversus perinei superficialis and the ventral band of the levator ani (fig 1) The smooth muscle of the perineal body was not identified histologically in this study It has been well described by Geiger ('54) as strands of the ventral longitudinal muscle of the rectum that turn ventrally decussate and pass into the smooth muscle of the labia and vestibule This tract of smooth muscle known as the *Afterschamband* in German veterinary literature has received no Latin name in the NA but is apparently homologous to m rectourethralis in the male Hayek ( 65) in his histological study of the smooth muscle of the human perineum suggested the term m rectoperinealis for the female He showed a predominance of smooth muscle

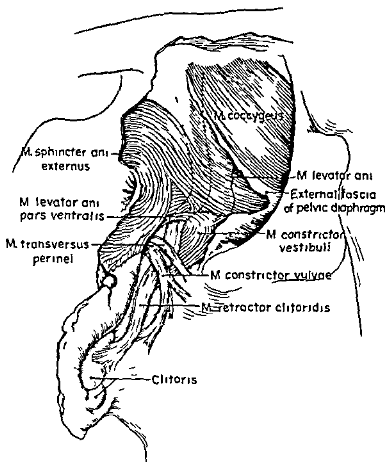



Fig 1 Perineal muscles of a cow exposed by removal of the deep fascia and retraction of th right labium Caudolateral aspect.

## MUSCLES AND FASCIAE

### *Regio perinealis*

The terms in table 1 were extracted and rearranged from a list adopted by the World Association of Veterinary Anatomists for publication in *Nomina anatomica veterinaria* The terms not applicable to this paper have been omitted and the order has been changed because the original typography identical with that of *Nomina anatomica* (NA Mitchell 61) gives the impression that the pelvic and urogenital diaphragms include all the structures in the anal and urogenital regions According to Gray ( 59) and Gardner et al ( 63) the pelvic diaphragm is composed only of the levator ani and coccygeus muscles and the fasciae investing them the urogenital diaphragm in man is composed of the transversus perinei profundus which does not occur in quadrupeds the sphincter urethrae and the fasciae on the inner (superior) and outer (inferior) surfaces of these muscles

Before the bovine perineum can be defined it is necessary to distinguish three concepts often confused the perineum the perineal region and the perineal body The perineum is the part of the body wall that covers the pelvic outlet and surrounds the anal and urogenital canals As a part of the body wall it has an appreciable thickness and its deep boundaries — those of the pelvic outlet — are not the same as its superficial boundaries — those of the perineal region (Gray 59) An anatomical region is a surface area without depth but the perineal region was considered a part of the trunk by Gardner et al ( 63) and the same idea was implied by the change from Perineum to Regio perinealis under Partes corporis in the NA (Mitchell 61) The meaning of the term perineum is often restricted in obstetrics to the fibromuscular mass between the anus and vulva but this is more properly called the perineal body or Centrum tendineum perinei (NA)

The deep boundaries of the perineum in the cow were the first coccygeal vertebra the sacrotuberal ligament the dorsal and ventromedial processes of the tuber ischiadicum and the ischial arch (There is no pubic arch in domestic mammals ) The superficial boundaries of the perineum or the boundaries of the perineal region had to be extended much farther ventrally than the ventral commissure of the labia to accommodate the perineal nerves and vessels in the area corresponding to that between the anus and the scrotum in the bull Therefore the perineal region in the cow was bounded by the base of the tail the sacrotuberal ligament (easily palpated) the dorsal and ventromedial processes of the tuber ischiadicum and a line from the tuber ischiadicum to the caudal attachment of the udder (fig 7) It could be

TABLE 1

*Nomenclature of muscles and fasciae*

| Perineum |
|---|
| Regio perinealis |
| Regio analis |
| Regio urogenitalis |
| Diaphragma pelvis |
| M levator ani |
| M coccygeus |
| Fascia diaphragmatis pelvis interna |
| Fascia diaphragmatis pelvis externa |
| M sphincter ani externus |
| Fossa ischiorectalis |
| Corpus adiposum fossae ischiorectalis |
| Canalis pudendalis |
| Fascia pelvis |
| F pelvis parietalis |
| Fascia obturatoria |
| F pelvis visceralis |
| Septum rectovaginale |
| Centrum tendineum perinei |
| Diaphragma urogenitale |
| M sphincter urethrae |
| M urethralis |
| M ischiourethralis |
| M bulboglandularis |
| Fascia diaphragmatis urogenitalis interna |
| Fascia diaphragmatis urogenitalis externa |
| M transversus perinei superficialis |
| M ischiocavernosus |
| M bulbospongiosus |
| M constrictor vestibuli |
| M constrictor vulvae |
| M retractor clitoridis |
| Fascia superficialis perinei |
| Fascia profunda perinei |

transverse processes of the first three coccygeal vertebrae Its lateral surface was related to the internal pudendal artery the branches of the pudendal nerve the lesser sciatic foramen and the fat in the ischiorectal fossa

*Fasciae of the pelvic diaphragm* (figs 1 5) The internal fascia of the pelvic diaphragm covered the medial surface of the coccygeus and levator muscles forming a part of the parietal pelvic fascia with which it was continuous cranially and dorsally Caudally it passed from the levator to the rectum where it was continuous with the visceral pelvic fascia At the caudal border of the coccygeus muscle it fused with the external fascia Ventrally it joined the external fascia at the ventral border of the levator and gave off the rectovaginal septum

The external fascia of the pelvic diaphragm covered the lateral surface of the coccygeus and levator muscles It was attached cranially to the deep face of the sacrosciatic ligament and dorsally to the fascia on the sacrococcygeus At the caudal border of the coccygeus it fused with the internal fascia of the pelvic diaphragm and joined the coccygeal fascia dorsal to the anus It was attached to the tendinous inscription of the levator and was continuous with the deep fascia on the external anal sphincter and constrictor vestibuli Ventrally it was continuous with the internal fascia at the ventral border of the levator gave origin to the caudal part of the urethral muscle and joined the urogenital diaphragm

The pelvic diaphragm in quadrupeds is difficult to visualize as such because the right and left halves are so oblique that they lie almost in sagittal planes

*M sphincter ani externus* The external anal sphincter completely encircled the anus (figs 3 4) Its most superficial fibers were radial and were attached to the skin Most of the fibers crossed ventral to the anus and continued into the opposite labium as the constrictor vulvae A few lateral fibers did not cross but passed into the labium on the same side

The cranial part of the external anal sphincter was variably fused with the terminal portion of the levator The dorsal band of the levator which met its mate over the anus sometimes appeared to belong to the anal sphincter An inconstant band of fibers took origin from the third coccygeal vertebra behind the coccygeus muscle and passed ventrally through the levator or across its deep surface to become continuous with the constrictor vestibuli This band when present may be interpreted either as a coccygeal insertion of the levator or as a paired cranial part of the anal sphincter like that of the horse

According to Bassett (65) the cranial part of the anal sphincter and the constrictor vestibuli in the sheep form a continuous muscle the deep perineal sphincter The functional concept may be useful but the new term is unnecessary and undesirable because the anal and vestibular components are homologous to muscles that have received separate names in other domestic animals

*Fossa ischiorectalis* The ischiorectal fossa (Godina 39) lay lateral to the anus where it formed a visible depression in old multipara (figs 4 7) It was a triangular pyramid with three faces a caudal base and a cranial apex In animals in normal condition it was filled with fat The medial wall was formed by the external fascia of the pelvic diaphragm and by the deep fascia on the constrictor vestibuli The laterodorsal wall was the sacrosciatic ligament and skin The lateroventral wall was the tuber ischiadicum and the obturator fascia The apex was at the origin of the coccygeus muscle from the deep face of the sacrosciatic ligament The base was covered by superficial fascia and skin The internal pudendal artery and vein and the pudendal nerve ran obliquely caudoventrad in the ventral groove near the apex of the fossa between the obturator fascia and the fascia on the coccygeus Laterally the distal cutaneous branch of the pudendal nerve passed along the medial surface of the tuber ischiadicum Medially the deep perineal nerve ran along the genital tract to the urogenital muscles (fig 5)

*Regio urogenitalis*

*M transversus perinei profundus* has not been demonstrated in quadrupeds with the possible exception of a muscle in the male goat called the ischiourethralis by Heinemann (37) A similar muscle oc

rather than tendinous tissue in the perineal center which has extensive connections in man and animals with the fasciae of the pelvic and urogenital diaphragms also shown by Hayek to have a large proportion of smooth muscle fibers

*Regio analis*

*M levator ani* (fig 3) The narrow tendon of origin was attached to the medial side of the sciatic spine near the lesser sciatic foramen It was fused laterally with the tendon of the coccygeus which was adherent to the medial surface of the sacrosciatic ligament (lig sacrospinotuberale) The levator was inserted by means of three main bands the fibers of which interpenetrated or were continuous with the fibers of the external anal sphincter The middle band about 3 cm wide was inserted into the lateral part of the sphincter The dorsal band about 1 5 cm wide blended with the sphincter dorsal to the anus where it was continuous with the contralateral muscle The ventral portion blended with the ventral decussation of the external sphincter A fourth inconstant insertion was attached to the coccygeal fascia (See cranial part of external anal sphincter )

The levator bore a transverse tendinous inscription (Geiger 54) where the fibers began to diverge to the insertion At the ventral end of the inscription the constrictor vestibuli originated from the ventral border of the levator and from the fascia on its surface The dorsal end of the inscription was attached to the coccygeal fascia The external fascia of the pelvic diaphragm was loosely attached to the lateral surface of the levator except at the tendinous inscription with which it was fused

The medial surface of the levator was related to the rectum and in the plane of the tendinous inscription to the retractor clitoridis The caudal rectal nerve lay on the medial surface of the caudodorsal part of the muscle The lateral surface was related to the coccygeus and to the ischiorectal fossa

Bassett ( 65) has raised again the question of the nomenclature of the levator ani in quadrupeds proposing that the coccygeus and levator be considered a single muscle the ischiococcygeus This concept and terminology should be rejected for the following reasons (1) The muscles are quite distinct in other animals the coccygeus is lateral to the levator its fibers take a different direction and it is separated from the levator by a septum Geiger's ( 56) description and illustrations show the two muscles to be separate in the sheep (2) The embryologic evidence presented by Bassett indicates that the coccygeus develops earlier than the levator (3) Bassett s main argument against the use of the term levator ani in ungulates is that two parts of the human levator the iliococcygeus and pubococcygeus are not present with the same origins and insertions as in man The human iliococcygeus is not directly attached to the ilium but originates in part from the spine of the ischium as in ungulates the insertion on the coccygeal fascia is much larger than in ungulates The difference is a quantitative one readily explained by the increased supportive function in the erect posture The human pubococcygeus originates from the pubis and is inserted in part on and around the external anal sphincter The difference in ungulates is that the muscle is smaller and the pubic origin is lost or transferred to the spine of the ischium According to Geiger ( 56) the pubic origin is represented in the sheep by the arcus tendineus which extends from the origin of the levator across the obturator internus to the pelvic symphysis The arcus tendineus was also described by Bassett ( 65 The anal insertion is primary in ungulates and this idea would be lost if the term ischiococcygeus were adopted (4) It is unfortunate that levator ani a gravity oriented term has been sanctioned by inclusion in the Basel Jena and Paris nomenclatures of human antomy (Kopsch 57) but since the function of the muscle as a part of the pelvic diaphragm is the same with regard to the long axis of the trunk in man and quadrupeds the name should be retained on functional as well as morphologic grounds

*M coccygeus* (figs 3 5) The common tendon of origin with the levator arose from the medial side of the sciatic spine and from the inside of the sacrosciatic ligament The muscle extended dorsocaudally crossing the lateral face of the levator obliquely and was inserted on the

separates m bulbospongiosus (m constric or vestibuli) from m urethralis

M urethralis in the cow covered the caudal half of the urethra the suburethral diverticulum and the lateral walls of the genital tract at the junction of the vagina vestibule (fig 3) It was 6 mm thick ventrally but thin at the lateral attachments The entire craniocaudal extent was about 9 cm Only the cranial 2 cm of the muscle was a true sphincter surrounding the urethra the rest of the muscle consisted of fibers that passed around the ventral surface of the urethra and suburethral diverticulum from one side of the genital tract to the other The vaginal segment of the muscle was 4 cm wide and the vestibular segment 3 cm wide extending back to the urogenital diaphragm The caudal part took origin from the fascia on the ventral border of the levator The muscle fibers became shorter cranially arising from the visceral pelvic fascia that extended ventrally from the rectovaginal septum onto the lateral wall of the vagina Thus the muscle formed a broad sling under the urogenital tract Its contraction would compress the urethra and suburethral diverticulum and constrict the mouth of the vagina It was covered by a tightly adherent tunic of visceral pelvic fascia 1–2 mm thick

M ischiourethralis was 6–10 cm long 3 cm wide flat and rectangular (figs 2 3) It took origin from the ischial arch and extended medioventrally to end in a flat thick aponeurosis which joined right and left muscles ventral to the vestibule The aponeurosis was related ventrally to the crura clitoridis and dorsally to the tendon of the constrictor vestibuli The cranial edge was continuous with the tunic on the caudal border of the ventral surface of the urethral muscle The deep surface of the ischiourethralis was related to the ischial arch the ischiocavernosus muscle and the internal fascia of the urogenital diaphragm The superficial surface was covered by the external fascia of the urogenital diaphragm

M bulboglandularis (Heinemann 37) is proposed as a general term for the striated muscle covering the bulbourethral gland or its female homologue the major vestibular gland This muscle has been described in the male of various species as a derivative of the urethralis ischiourethralis or bulbospongiosus and has been given different names in each instance (Brauell 1868) It is also subject to individual variation The characteristic feature of the muscle is not its origin but its relation to the gland

The bulboglandularis was present in the cow in varying degrees of development It was usually a band from the urethral muscle about 5 mm wide that curved around the caudal dorsal and cranial borders of the major vestibular gland (fig 3) In some specimens it originated from fibers of the constrictor vestibuli that penetrated the external fascia of the urogenital diaphragm

*Fasciae diaphragmatis urogenitalis* In the cow the urogenital diaphragm was a strong semicylindrical sheet extending from the ischial arch inward and cranially to the ventral and lateral walls of the vestibule It was partially separable into two layers of fascia which enclosed the ischiourethralis the rudimentary ischiocavernosus and the major vestibular gland with the bulboglandularis (figs 2–5) Although m urethralis must be included in m sphincter urethrae and therefore in diaphragma urogenitalis in table 1 for comparative reasons it is actually located cranial to the urogenital diaphragm in quadrupeds because of the elongation of the pelvic urethra The ischiocavernosus is associated with the urogenital diaphragm only in the rudimentary female form The diaphragm would appear to anchor the genital tract to the pelvic outlet against the forward pull of the gravid uterus and when everted during parturition against the expulsive efforts

Geiger (56) in his description of the perineum of the sheep and goat said that the urogenital diaphragm was formed by m constrictor vestibuli and m transversus perinei superficialis Such a diaphragm would not be homologous to the human urogenital diaphragm and should not be so named These muscles are superficial to the diaphragm

The external fascia of the urogenital diaphragm faced dorsally and caudomedially It was attached to the ischial arch along the caudal border of the ischiourethralis

curs as a variation in the ram and bull but as these muscles were described without reference to the urogenital diaphragm, they have not been shown to be homologous to the human transverse perineal muscle, which extends from the ischium to the perineal center between the fasciae of the urogenital diaphragm

*M sphincter urethrae* The human female sphincter urethrae is a flat transverse, striated muscle in the ventral part of the urogenital diaphragm It originates from the inferior ramus of the pubis and most of its fiber bundles are inserted into the lateral wall of the vagina but a few pass ventral to the urethra and a few pass between the urethra and the vagina (Gardner et al 63) The bundles surrounding the urethra are better developed in the male

No comparative study of the components of this muscle has been found Because of the greater length of the pelvic urethra and the meager development of the urogenital diaphragm in quadrupeds the sphincter is more important than the transverse component In veterinary anatomy the parts have been described as three separate muscles m urethralis m ischiourethralis and m bulboglandularis Much confusion has arisen from a failure to distinguish between the smooth muscle in the urethral wall and the striated m urethralis the smooth muscle is not named in NA Another source of confusion is the failure to recognize the urogenital diaphragm which

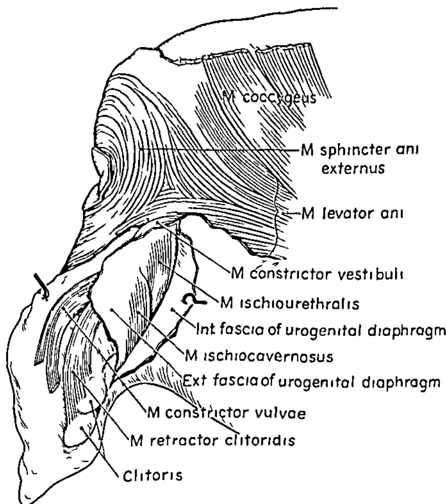



Fig 2 Perineal muscles of a cow in lateral view after removal from the pelvis The urogenital diaphragm is split the labium is drawn back and the deep bands of the constrictor vulvae have been severed from their insertions on the fascia below the ischial arch

ourse of its fibers to the insertion on the he crus clitoridis The aponeurosis of in ertion could be separated by dissection rom the overlying aponeurosis of the schiourethralis

*M constrictor vestibuli* (figs 1 3 4 6) The origin was from the ventral border of he levator ani at the tendinous inscription and from the fascia covering the levator Variable fibers from other parts of the leva tor also joined the constrictor The mus cle became thin ventrally and ended in a flat tendon which passed under the floor of he cranial end of the vestibule to meet the endon of the opposite muscle This was over the caudal end of the pelvic sym physis Contraction of the paired muscles raises a U shaped ridge inside the vesti bule according to Geiger (54) When fixed *in situ* the upper part of the muscle was triangular in cross section The medial surface was related to the vestibule and the m retractor clitoridis the caudolateral surface to the fat in the ischiorectal fossa and the cranial surface to the urogenital diaphragm which contained the major vestibular gland If the muscle exerts any pressure on the gland it does so by press ing against the urogenital diaphragm

Bassett (65) basing the argument on dissections of the ewe denied that the con strictor vestibuli is homologous to the bul bospongiosus Comparison to human anat omy is not helpful here because the ex tremely short human vestibule leaves no room for the development of a separate constrictor vestibuli However in domestic animals a striated muscle surrounding the urogenital sinus just caudal (external) to the urogenital diaphragm is certainly homologous to the cranial part of the male bulbospongiosus

*M constrictor vulvae* (figs 1–7) was continuous with the external anal sphinc ter at the decussation The superficial fi bers spread out subcutaneously in the labia They were thin and dispersed in the fascia and many of them were attached to the skin The deep part of the muscle consisted of three or four bands which had a common origin from the anal sphincter and diverged ventrally on the fascia cover ing the semimembranosus muscle below the ischial arch

*M retractor clitoridis* (figs 1–3 6) This smooth muscle originated from the second and third or third and fourth coccygeal vertebrae and passed around the lateral surface of the rectum covered by the ter minal part of the levator Variable strands of smooth muscle joined the retractor from origins on the longitudinal muscle of the rectum the external anal sphincter and the levator ani According to Basset (61) the part between the rectum and the ver tebrae may fail to develop in the sheep It was present in all of the bovine specimens in this study At the ventral border of the levator the retractor crossed obliquely the medial surface of the constrictor vestibuli and ran ventrally between the constrictors of the vestibule and vulva Unlike the equine retractor (Habel 53) the bovine had no loop connecting right and left re tractors through the perineal body The termination was variable it divided into several strands one or more of which were inserted on the corpus clitoridis Other branches were attached to the wall of the vestibule or to the fascia on the semimem branosus The muscle in the ewe contained some scattered striated fibers (Bassett 61)

*Fasciae perinei*

*Fascia superficialis perinei* (fig 7) The superficial fascia was mingled with the subcutaneous fibers of the external anal sphincter and the constrictor vulvae It was continuous with the fat that filled the ischiorectal fossa The condensation of superficial fascia in the tail fold was at tached to the deep fascia on the caudal border of the coccygeus and on the lateral border of the external sphincter Else where the superficial fascia was readily separable from the deep fascia that covered the perineal muscles

*Fascia profunda perinei* (fig 5) The deep perineal fascia covered the circular fibers of the external anal sphincter the deep part of the constrictor vulvae and the constrictor vestibuli and extended to the scrotum or udder as a narrow zone contin uous with the caudal femoral fascia on either side It included the external fascia of the pelvic diaphragm and the fascia of the urogenital diaphragm

and was continuous here with the caudal femoral fascia It passed across the superficial surface of the ischiourethralis and around the cranial border of the constrictor vestibuli to its attachment on the wall of the vestibule It covered the caudal surface of the major vestibular gland

The internal fascia of the urogenital diaphragm faced ventrally and craniolaterally It arose from the ischial arch and the obturator fascia passed across the deep surface of the ischiourethralis and the cranial surface of the major vestibular gland and attached to the vestibule at the caudal border of the urethral muscle It was continuous here with the visceral pelvic fascia on the surface of the muscle

Dorsally both layers of the urogenital diaphragm were attached to the perineal center and to the fasciae of the pelvic diaphragm at the ventral border of the levator

Ventral to the vestibule the fasciae of the urogenital diaphragm were fused with the tendons of the ischiourethralis and ischiocavernosus, and formed a strong connection between the fascia on the ventral surface of the urethral muscle and the deep perineal fascia ventral to the vulva An oval foramen with strong tendinous borders perforated the fascia at the caudal end of the pelvic symphysis Through it passed the perineal vein the nerve of the clitoris the mammary branch of the pudendal nerve and the internal pudendal artery after leaving the pelvic cavity through the space between the crura clitoridis and the ischial arch

*M transversus perinei superficialis* was a rudimentary band about 5 mm wide which extended from the decussation of the external anal sphincter for a variable distance toward the tuber ischiadicum in the superficial fascia (figs 1 4)

*M ischiocavernosus* (figs 2 3) was 6 cm long 2 cm wide and very thin It lay on the medial part of the ischial arch covered by the ischiourethralis muscle It was distinguished from the latter by the oblique

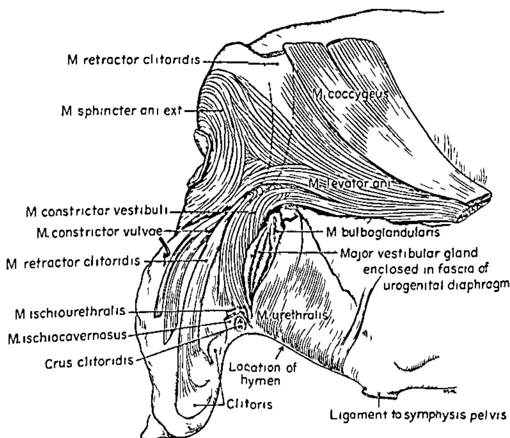



Fig 3 Same specimen as figure 2 after removal of most of the urogenital diaphragm and removal of the visceral pelvic fascia from the urethral muscle

*Nn splanchnici pelvini* (fig 5)

The largest pelvic splanchnic nerve was formerly called the middle hemorrhoidal nerve in the horse (Ellenberger and Baum 43) but the NA term should be adopted In the cow it originated from sacral nerves 2 to 4 by means of the same branches that joined to form the pudendal nerve The pelvic splanchnic nerve then left the medial surface of the proximal end of the pudendal and ran ventrally on the rectum to join the autonomic plexuses on the pelvic viscera Smaller pelvic splanchnic nerves were given off from the pudendal near the vestibule from the deep perineal nerve from the muscular branches and from the caudal rectal nerves

*Rami musculares* (fig 5)

In man (Gray 59) and dog (Miller et al 64) the branches to the levator ani and coccygeus extend directly from the sacral plexus to the muscles In the horse (Habel 53) and pig (Reimers 13) the muscular branches are combined with the caudal rectal nerve

In the cow the branches to the levator ani and coccygeus muscles were usually combined in a single nerve which originated from the fourth sacral nerve with a smaller contribution from S 3 via the pudendal Before passing to the medial surface of the coccygeus the muscular branch gave a ramus communicans to the distal cutaneous branch of the pudendal Four variations from the usual pattern have been demonstrated (1) Both muscular branches were incorporated in the pudendal leaving it just before the pudendal passed lateral to the coccygeus When the muscular branches ran with the pudendal wholly or in part an unusually large branch of S 4 joined the pudendal at its origin (2) Both muscular branches were incorporated in the caudal rectal nerve This combination was formerly called the middle hemorrhoidal nerve in the ox (Reimers 13) The reasons for eliminating the latter term are given in the discussion of *N rectalis caudalis* When the muscular branches were separate from the pudendal they always gave off a communicating branch to the distal cutaneous branch of the pudendal (3) One muscular branch came from the pudendal and one from the caudal rectal (4) One muscular branch was separate and the other was combined with the pudendal or caudal rectal

*N pudendus* (figs 5–7)

The origin was from sacral nerves 2 to 4 with the largest contribution from S 3 The pudendal nerve ran ventrocaudally on the medial surface of the sacrosciatic ligament dorsal to the internal iliac artery From the medial side near its origin it gave off a large pelvic splanchnic nerve It often contributed to the muscular branches to the coccygeus and levator ani Near the lesser sciatic foramen it gave off the proximal and distal cutaneous branches As it crossed the foramen it received the medial branch of the caudal cutaneous femoral and gave off the deep perineal nerve Thus four parallel nerves were seen crossing the lesser sciatic foramen craniocaudally on the lateral surface of the coccygeus and levator muscles (fig 5) From dorsal to ventral they were the proximal cutaneous branch distal cutaneous branch deep perineal and the continuation of the pudendal The latter turned caudomedially between the vagina and the pelvic floor accompanied by the internal pudendal artery and vein It gave small branches to the vestibule and ended as the dorsal nerve of the clitoris after giving off the mammary branch

*Ramus cutaneus proximalis* (figs 5 7) emerged through the dorsal part of the lesser sciatic foramen or through the sacrotuberal ligament near the dorsal process of the tuber ischiadicum It turned ventrad and supplied the skin over the semitendinosus muscle an area innervated by the caudal cutaneous femoral nerve in other species Rarely the communication from the muscular branch joined the proximal instead of the distal cutaneous branch

*Ramus cutaneus distalis* (figs 4 5 7) After receiving the communication from the muscular branch the distal cutaneous branch coursed caudally through the ischiorectal fossa on the medial surface of the tuber ischiadicum about midway between the dorsal and ventromedial processes of the tuber (figs 4 7) It turned ventrally over the caudal surface of the tuber The

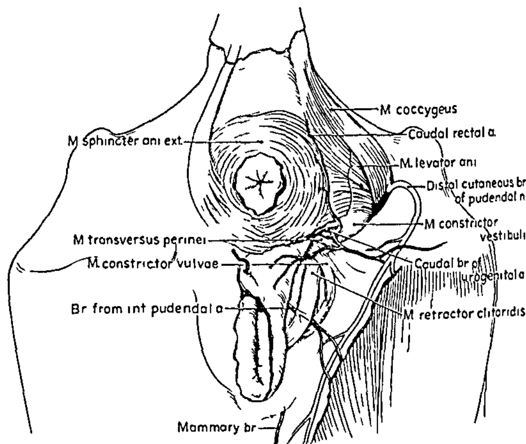



Fig 4 Perineal arteries of a cow in which the cutaneous branches to the region lateral and ventral to the vulva and to the mammary gland were supplied by the internal pudendal These branches are supplied less frequently by the urogenital (vaginal) artery (fig 6)

## NERVES

### *N cutaneus femoris caudalis* (table 2 fig 5)

Because most of the caudal thigh region was supplied by the cutaneous branches of the pudendal nerve the caudal cutaneous femoral nerve was very small (Habel 56) It arose from the dorsal border of the sciatic nerve on the lateral surface of the sacrosciatic ligament at a variable distance from the greater sciatic foramen to a point half way between the sciatic foramina The nerve ran caudally dorsal to the sciatic nerve on the lateral surface of the sacrosciatic ligament or on the origin of the deep gluteal muscle At the lesser sciatic foramen it divided into two branches The medial branch passed into the foramen between the caudal gluteal and internal pudendal arteries and joined either the pudendal nerve or its deep perineal branch or both According to Schreiber (56) the lateral branch may be absent It may join the proximal cutaneous branch of the pudendal or it may penetrate the caudal border of the biceps to supply the skin in an area parallel and lateral to the proximal cutaneous branch of the pudendal nerve

TABLE 2
*Nomenclature of nerves*

| |
|---|
| Plexus sacralis |
| N cutaneus femoris caudalis |
| Nn splanchnici pelvini |
| Rami musculares |
| N pudendus |
| Ramus cutaneus proximalis |
| Ramus cutaneus distalis |
| N perinealis superficialis |
| Nn labiales |
| N perinealis profundus |
| Ramus mammarius |
| N dorsalis clitoridis |
| Nn rectales caudales |

ran caudally on the lateral surface of the coccygeus and levator ani muscles The medial branch of the caudal cutaneous femoral nerve joined the pudendal or the deep perineal nerve or both near the origin of the latter The deep perineal gave branches to the urethral muscle the vagina and the major vestibular gland It then perforated the urogenital diaphragm dorsal to the gland and passed medial to the origin of the constrictor vestibuli which it innervated The nerve turned dorsally on the deep surface of the terminal bands of the levator and innervated them and the lateral margin of the external anal sphincter A twig entered the adjacent caudal surface of the retractor clitoridis and communicated through the muscle with the caudal rectal nerve Thus the deep perineal nerve innervated the urethral muscle and constrictor vestibuli and part of the external anal sphincter and levator ani

The term deep perineal nerve was introducted into veterinary anatomy by Larson and Kitchell ( 58) who found that it innervated the homologous muscles in the male plus the ischiocavernosus In man it is called the deep branch of the perineal nerve (Gray 59) but is not listed in the NA It was called the perineal branch of the pudendal in the mare (Habel 53) In the dog it arises as a series of small branches from the pudendal (Miller et al 64)

*Ramus mammarius* (figs 5–7) This branch turned ventrally on the deep perineal fascia accompanied by the mammary branch of the perineal artery and the perineal vein and ran to the skin of the caudal surface of the udder In some specimens a fine communicating branch joined the overlying superficial perineal nerve In adult cows the perineal vein was often tortuous and its loops partially concealed the nerve and artery

The mammary branch of the pudendal nerve (Habel 49) was called the perineal nerve by St Clair ( 42) and the superficial perineal nerve by Larson and Kitchell ( 58) who traced it in the male to the cranial aspect and median septum of the scrotum and to the prepuce Ramus mammarius in the female and ramus preputialis et scrotalis in the male are preferable terms for the following reasons (1) These nerves do not supply the perineal region which does not include the udder cranial surface of the scrotum or prepuce (2) These nerves are not superficial in the perineal region they do not supply the skin there (3) In the ox the superficial nerves of the perineal region including the caudal scrotal nerve as described by Larson and Kitchell ( 58) are supplied by the distal cutaneous branch of the pudendal — not one of the terminal branches

*N dorsalis clitoridis* (fig 5) supplied the clitoris and sometimes gave a branch to the vestibule Right and left nerves of the clitoris and mammary branches lay on the pelvic symphysis and emerged by passing between the crura clitoridis and the symphysis then through the foramen in the deep perineal fascia

*Nn rectales caudales* (figs 5 6)

One or two caudal rectal nerves originated from the fourth or fourth and fifth sacral nerves They ran caudally between the rectum and the coccygeus supplying small pelvic splanchnic nerves to the rectum and sometimes giving off one or both muscular branches to the coccygeus and levator ani The main caudal rectal nerve is easily exposed by dissection in the caudal angle between the coccygeus and levator (fig 6) On the deep surface of the levator cranial to the retractor clitoridis the nerve divided into several branches (fig 6) A dorsal branch went through the retractor clitoridis to the external anal sphincter and the dorsal band of the levator A middle branch passed through or medial to the retractor clitoridis and deep into the perineal center where it entered the cranial surface of the decussation of the external anal sphincter Some fibers reached the mucosa of the roof of the vestibule and others ran deep in the labium

The largest or ventral branch passed through the retractor clitoridis and innervated it Coursing medial to the origin of the constrictor vestibuli it gave twigs to that muscle and the adjacent part of the levator and communicated with the deep perineal nerve It crossed obliquely the lateral surface of the retractor clitoridis in the labium and emerged through the deep part of the constrictor vulvae to end in the superficial fascia of the labium The termi

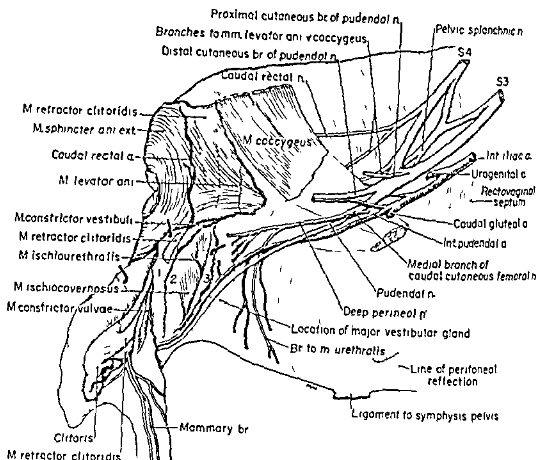



Fig 5 Lateral view of the pelvic viscera of a cow the superficial fascia of the labium is retracted by a hook 1 external layer of deep perineal fascia 2 external fascia of urogenital diaphragm 3 internal fascia of urogenital diaphragm The arterial pattern illustrated is the more usual one in which the internal pudendal artery supplies the region lateral and ventral to the vulva Another pattern is shown in figure 6

first branch was given off on the tuber ischiadicum and ran medially into the ischiorectal fossa Another small medial branch was given off below the tuber at the level of the dorsal commissure of the labia At the same level the trunk divided into three main branches The medial branch gave two or three branches to the labium and ended by ramification on the midline over the perineal vein It sometimes communicated with the mammary branch of the pudendal nerve The middle branch supplied the skin over the semimembranosus and extended to the skin between the udder and thigh The lateral branch ran along the septum between the semimembranosus and semitendinosus

The branches to the perineal region and labia are the superficial perineal and labial nerves which are derived from the pudendal and emerge from the ischiorectal fossa as in man and dog *In the horse however* the caudal rectal nerve after receiving a communicating branch from the pudendal supplies the superficial perineal nerves through the ischiorectal fossa (Habel 53) *In the cow* the caudal rectal nerve also reaches the labium but independently by passing medial *to the levator* ani (fig 5) This *termination* was called the perineal nerve by Reimers ( 13) it is best left unnamed (Larson and Kitchell 58)

In the bull the *superficial perineal* nerves (*medial branches of the right* and *left distal cutaneous branches*) joined to form the caudal scrotal nerve (Larson and Kitchell 58)

*N perinealis profundus* (figs 5 6) This nerve arose from the dorsal border of the pudendal at the lesser sciatic foramen and

ontinued caudally giving small variable branches to the obturator muscle and divided at the cranial border of the lesser sciatic foramen into its terminal branches the caudal gluteal and internal pudendal arteries (see table 3)

*A urogenitalis*

This term (Miller 47) has been in use for many years in veterinary anatomy to designate the large artery that supplies the intrapelvic part of the urogenital system It is partially homologous to the human vaginal artery but supplies a much larger area in ruminants and swine Barone (57) Preuss (59) and Vollmerhaus ('64) have adopted the term vaginal artery for use in domestic animals but they do not agree on the names of its branches

TABLE 3

*Nomenclature of arteries*

A iliaca interna

- A umbilicalis
- A uterina
- A iliolumbalis
- A glutea cranialis
- A urogenitalis (A *vaginalis*)
  - Ramus cranialis
    - R urethralis
    - A vesicalis caudalis
    - A ureterica
    - R uterinus
  - Ramus caudalis
    - Rami vaginales
    - Rami rectales medii
    - R glandularis
    - R vestibularis
    - A rectalis caudalis
    - A perinealis
      - Rr labiales
      - Rr perineales dorsales
      - (Rr perineales ventrales)
      - (R mammarius)
- A glutea caudalis
- A pudenda interna
  - R vaginalis
  - R vestibularis
  - R glandularis
  - A profunda clitoridis
  - A dorsalis clitoridis
  - Rr perineales ventrales
  - R mammarius

After a short course to the lateral surface of the vagina the urogenital artery divided into cranial and caudal branches

*Ramus cranialis* was the larger It supplied branches to the urethra bladder and ureter and was continued as the uterine branch The latter was the largest branch of the urogenital artery in the parous cow and was formerly called the caudal uterine artery Vollmerhaus ('64) designated the cranial branch of the urogenital as the continuation of the vaginal artery and the caudal branch as the perineal artery This is not helpful to understanding because the caudal branch is the one that supplies most of the vagina

*Ramus caudalis* (figs 4 6) ran back along the dorsolateral surface of the vagina giving small branches to the vagina and rectum It passed medial to the dorsal end of the major vestibular gland and gave off a branch to the gland A small branch ran dorsally on the deep surface of the retractor clitoridis and the caudal branch crossed the medial surface of the muscle After giving a branch to the vestibule the caudal branch emerged between the retractor clitoridis and the ventral branch of the levator and ended by dividing into the caudal rectal and perineal arteries Because the caudal branch of the urogenital in ruminants and swine replaces a part of the internal pudendal artery of man dog and horse it is not homologous to the human vaginal artery

*A rectalis caudalis* (figs 4–7) ran dorsally on the lateral border of the external anal sphincter and ended in the tail fold The nomenclature used by Vollmerhaus ('64) for the middle and caudal rectal arteries is that of the Jena NA 1936 (Kopsch '57) in which they were listed as a rectalis caudalis and a analis

*A perinealis* gave off a lateral branch to the region of the tuber ischiadicum and turned ventrally in the labium between the retractor clitoridis and constrictor vulvae In two-thirds of the cows dissected the perineal artery derived from the urogenital terminated in the labium (figs 4 5) In one third it supplied the cutaneous branches to the perineal region lateral and ventral to the vulva and the branch to the mammary gland (fig 6)

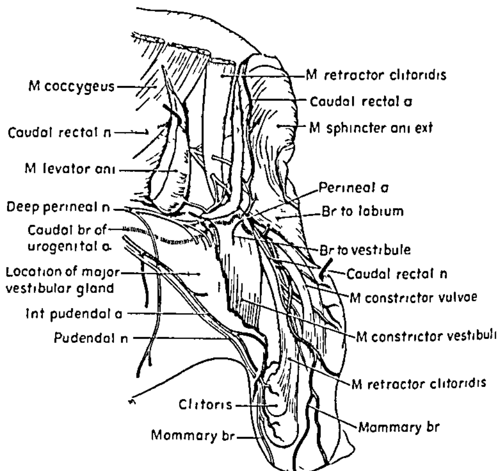



Fig 6 Lateral view of the pelvic viscera of a cow dissected to show the relation of the caudal rectal and deep perineal nerves The arterial pattern shown here in which the cutaneous branches to the region lateral and ventral to the vulva and the branch to the mammary gland are supplied by the urogenital (vaginal) artery occurs less frequently than that shown in figure 5

nal fibers of all three branches of the caudal rectal nerve may reach the skin but this was not demonstrated by dissection Larson and Kitchell (58) showed that the nerve in the male was sensory and that it was also motor to the coccygeus levator ani external anal sphincter and retractor penis

For many years the combination of a caudal rectal nerve with the muscular branches to the coccygeus and levator ani was called in the ox the middle hemorrhoidal nerve The caudal homorrhoidal (rectal) nerve was said to be often absent (Reimers 13 Habel 49 Larson and Kitchell 58) This was contrary to the nomenclature of the same nervous elements in man dog and horse It is better from a comparative and didactic standpoint to drop the term middle hemorrhoidal and to follow the NA in recognizing only nn rectales caudales in the plural At least one caudal rectal nerve is always present Lassoie (58) described it as one nerf *anal* which terminated in two parallel branches running caudally on the rectum

## ARTERIES

### *A iliaca interna* (fig 5)

The first branch of the internal iliac artery was the common trunk of the uterine and umbilical arteries After giving off the iliolumbar and cranial gluteal arteries the internal iliac coursed along the medial side of the ventral border of the sacrosciatic ligament The urogenital artery usually originated near the ischiatic spine although it sometimes arose more proximally or as far distally as the internal pudendal artery The internal iliac

gave off a large branch that supplied the cutaneous branches to the perineal region lateral and ventral to the vulva and the small cutaneous branch to the mammary gland In one third of the cows this area was supplied by the urogenital artery Different arterial patterns may occur on right and left sides of the same cow

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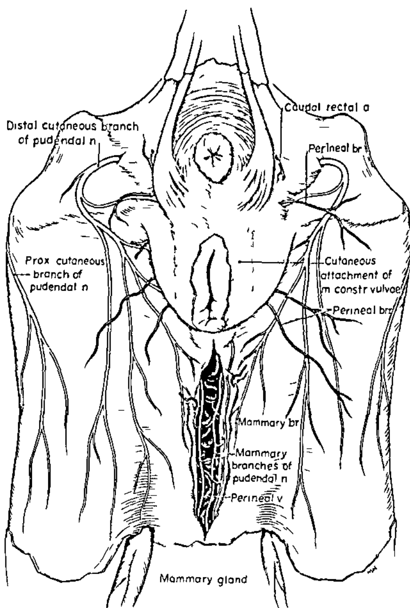



Fig 7 Perineal region of a cow after removal of the skin The superficial fascia ventral to the vulva is incised and retracted The arteries lateroventral to the anus are supplied by the urogenital (vaginal) artery The arteries lateral and ventral to the vulva are usually supplied by the internal pudendal but may be from the urogenital

*A pudenda interna*

After giving a muscular branch to the coccygeus and levator ani the artery ran caudally on the lateral surface of these muscles (fig 5) A branch accompanied the distal cutaneous branch of the pudendal nerve to the ischiorectal fossa Branches were given off to the vagina vestibule and major vestibular gland as the trunk inclined ventrally on the internal surface of the urogenital diaphragm accompanied by the internal pudendal vein and the pudendal nerve Right and left vessels and nerves converged on the ventral surface of the vestibule and the veins joined to form one perineal vein After emerging between the crura clitoridis and the pelvic symphysis and through the foramen in the deep perineal fascia the internal pudendal artery supplied the deep artery of the clitoris to the crus and ended as the dorsal artery of the clitoris In two-thirds of the cows dissected the internal pudendal after perforating the deep fascia

# ıne Structure of the Surface of Mouse Hepatic Cells

TREVOR HEATH AND STEVEN L WISSIG

*Department of Veterinary Preclinical Sciences University of Melbourne Parkville Victoria Australia and Department of Anatomy University of California San Francisco Medical Center San Francisco California*

*ABSTRACT* Mouse hepatic cells appear in thin sections as polygons with six or more sides The plasma membrane covering these sides may contact either bile canaliculi the narrow intercellular space the space of Disse or extensions of the space of Disse between adjacent cells The plasma membrane covering microvilli in bile canaliculi and the space of Disse is thicker than that in contact with the narrow intercellular space Bile canaliculi which contact about 6% of the perimeter of each cell are each separated by tight junctions from the narrow intercellular space This space contacts more than one half the perimeter of each cell and is about 220 A in width It is continuous around the occasional studlike junctions which occur but is interrupted at frequent intervals by circumscribed tight junctions and occasionally by desmosomes The narrow intercellular space is in free communication through the space of Disse with the plasma space An interstitial fluid space separate from the plasma space does not occur in the liver lobule Protein molecules from plasma enter hepatic cells in both coated and pinocytotic vesicles These vesicles are derived from invaginations of the plasma membrane that borders the narrow intercellular space and the spaces between microvilli in the space of Disse Pinocytotic vesicles may also incorporate fat droplets into hepatic cells

Segments of the perimeter of parenchymal cells located in the interior of a hepatic lobule border one of three types of extracellular space a lumen of a bile canaliculus a perisinusoidal space of Disse or a narrow intercellular space separating adjacent hepatic cells The plasma membrane and adjacent cytoplasm along each type of space have specific morphologic characteristics peculiar to that region and descriptions of some of these have already appeared in the literature (Fawcett 55 David 61 Biava 64)

Bile canaliculi in the mouse rat guinea pig and man are most often limited by segments of the plasma membrane of two and less often of three or more hepatic cells (David 61 Biava 64) The plasma membrane which limits the canaliculi at least in man is 100–110 A thick but the thickness and density can vary depending on the procedures used to prepare the specimens for examination (Biava 64) The cytoplasm which borders each canaliculus contains a fine filamentous meshwork and strands from this meshwork extend into microvilli which project into the lumen (Wood 61) Minute tubules 70–90 A in diameter have also been described in canalicular microvilli in the rabbit liver (David 61)

There is some disagreement about the degree of continuity between the lumen of the canaliculi and the remainder of the extracellular space In an early series of reports Rouiller (54 56) stated that bile canaliculi communicate freely with the space of Disse As this statement has not been substantiated and as it cannot be reconciled with the known facts of liver physiology it seems likely that the author confused some extensions of the space of Disse for bile canaliculi Later Ashworth and Sanders (60) concluded that a cleft about 100 A wide separated adjacent hepatic cells at the margins of canaliculi They believed that this was just wide enough to allow water ions and small molecules to pass from the space of Disse into canaliculi but was too narrow to allow the passage of proteins More recently Farquhar and Palade (63) showed that the plasma membranes of adjacent hepatic cells in the rat and guinea pig fuse to form an apparently impermeable tight junction where they meet at the margins of a canaliculus These tight junctions exist as a continuous band (*zonula occlu*

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*dens*) along the length of the margins of all canaliculi and should effectively seal off the canalicular lumen from the adjacent intercellular space Farquhar and Palade (63) described two other components of a junctional complex at the lateral margins of the canaliculi These components the intermediate junction (*zonula adhaerens*) and the desmosome (*macula adhaerens*) were believed to act as intercellular attachment devices The junctional complex at the margins of human bile canaliculi consists of essentially the same components (Biava 64)

The segments of the hepatic cell surface which face the space of Disse and its extensions are covered by numerous microvilli (Rouiller 54) The cytoplasm immediately adjacent to the space of Disse contains many small vesicles and some of these vesicles appear to be connected to the space of Disse by tubular invaginations of the plasma membrane (Rouiller 54 Novikoff and Essner 60 Bruni and Porter 65) It has been suggested that these vesicles are pinocytotic in nature and that they are important in the uptake of materials by hepatic cells (Roth and Porter 62 Bruni and Porter 65) The observation of Hampton (58) that colloidal particles introduced into the space of Disse are incorporated into these vesicles seems to support this view

Over much of their perimeter neighboring hepatic cells face each other across a narrow intercellular space This space varies in width and is interrupted at intervals by various types of junctions between the adjacent cells (Fawcett 55 Ashworth and Sanders 60 Hampton 60 64 David 61) Small dense droplets have been observed in the space (Cossel 62) and Hampton (64) described small vesicles in the adjacent cytoplasm

The extracellular spaces in other tissues are believed to contain interstitial fluid which drains to the bloodstream in the lymphatics Although the liver produces relatively large volumes of lymph little is known of its origin Terminal lymphatics have not been described within the hepatic lobule and the relative contributions made by lobules and by periportal areas to the hepatic lymph have not been determined (Brauer 63) In this connection it would be interesting to determine whether a true extravascular interstitial fluid space exists within the hepatic lobule and serves as a reservoir from which lymph drains in a manner analogous to its drainage from other tissues

Although such topics as the differences in structure between different regions of the surface of hepatic cells the nature of the junctions between neighboring hepatic cells and the existence of a true interstitial fluid space have been treated incidentally and sporadically by several papers in the literature, they have not been systematically investigated for their own sake The present paper presents detailed quantitative and morphologic data concerning the entire surface of parenchymal cells of the mouse liver and also contains a description of the structure and location of various types of intercellular junctions along their perimeter In addition ferritin was introduced into the circulating blood to serve as a tracer so that the accessibility of extravascular spaces within the lobule to constituents of plasma could be examined

## MATERIALS AND METHODS

Mice of either the Cal A or the C3H strain were allowed free access to food until the experiments were started in the mornings The mice were anesthetized with pentobarbitone sodium A midline incision was made through the abdominal wall and thin strips of liver were removed into a drop of chilled fixative The strips were cut into small blocks which were immersed in fixative In some experiments fixation was initiated by dripping chilled fixative onto the surface of the liver for ten minutes before a thin strip of fixed tissue was removed cut into blocks and immersed in fixative

Some mice received an intravenous or intraperitoneal injection of a 10% ferritin solution before the liver specimens were collected Prior to injection the solution of ferritin was dialyzed against sodium versenate to remove traces of cadmium (Farquhar Wissig and Palade 61) Specimens of liver were removed up to 80 minutes after 0 5 ml of ferritin solution was injected into the tail vein of unanesthetized mice or into the external jugular

-in of anesthetized mice or 150 minutes ,ter 1 0 ml was injected into the peri neal cavity of unanesthetized mice

The blocks of liver which were collected each experiment were all fixed for 2 hours in buffered solutions of either mium tetroxide or glutaraldehyde One . 2% solutions of osmium tetroxide were iffered to a pH of 7 5 with either acetate -ronal (Palade 52) phosphate (Mil ing 62) or s-collidine (Bennett and Luft 9) Redistilled glutaraldehyde was buf -red to pH 7 5 with either cacodylate or hosphate (Sabatini Bensch and Barrnett ;3)

The blocks were subsequently dehy ,rated in acetone and embedded in either iraldite or Epon (Luft 61) Some of the ,locks were immersed in a solution of 1% otassium permanganate in acetone for ive minutes during dehydration (Parsons 31)

Thin sections were mounted on grids ,overed with formvar that had been sta ilized with carbon and were stained with both uranyl acetate (Watson 58) and a ead salt (Millonig 61 Reynolds 63) The sections were examined with a Sie mens Elmiskop 1

*Quantitative data*

In the first series measurements were made of the total perimeter of hepatic parenchymal cells and of the percentage of the perimeter which bordered bile can aliculi the space of Disse and its exten sions and the narrow intercellular space A flexible ruler was used to make these measurements on cells magnified 6 400 × that had been sectioned through the ap-proximate center of their nuclei It is pos sible that some of the cells contained more than one nucleus and in this case the plane of section may not have passed through the equator of the cell In seg ments of the surface coated with micro-villi the total length of the plasma mem brane could not be accurately measured at this magnification For this reason the linear extent of the surface bordering bile canaliculi was measured as the distance between the luminal extremities of the tight junctions at the canalicular margins (line *A* fig 1a) and that bordering the space of Disse and its extensions was measured across the bases of the micro-villi (line *A* fig 1b) Hepatic cells ap-peared polygonal in outline when viewed in equatorial section and the number of sides forming their perimeter as well as the number of bile canaliculi desmosomes and stud like processes at their surface was recorded (figs 2–4) These measure ments were all made on 22 cells from vari ous regions of hepatic lobules from four mice

In the second series the increase in length of the plasma membrane resulting from the presence of microvilli along seg ments of the cell surface was estimated on micrographs with a final magnification of 56 000–84 000 × Within bile canalic uli the total length of the plasma mem brane covering microvilli isolated profiles of microvilli and intermicrovillous spaces

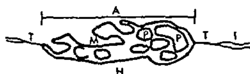



Fig 1a Diagram of bile canaliculus showing method used to estimate the increase in length of plasma membrane resulting from the pres-ence of microvilli The total length of the plasma membrane within the canaliculus (thick line) was compared with the length of the long axis of the canaliculus (line *A* ) *Abbreviations* *H* hepatic cell *M* microvillus *T* tight junction at margin of canaliculus I intermediate junction *P* isolated profile of microvillus

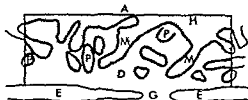



Fig 1b Diagram of hepatic cell surface fac ing the space of Disse showing method used to estimate the increase in length of the plasma membrane resulting from the presence of micro-villi The total length of hepatic plasma mem brane within the stippled area (thick line) was compared with the linear extent of the cell sur face upon which the microvilli are located (line *A* ) *Abbreviations* *H* hepatic cell *M* micro-villi *P* isolated profiles of microvilli *E* sinu soidal lining cell *G* gap between sinusoidal lin ing cells

(thick line fig 1a) was measured with a flexible ruler and compared with the linear extent of the surface bordering the canaliculi (line *A* fig 1a) Similar measurements were made within the space of Disse A line was drawn across the bases of the microvilli and at either end a perpendicular line was drawn to intersect the plasma membrane of the endothelial cell lining the sinusoid The total length of the hepatic plasma membrane within the roughly rectangular area defined by these three lines and the plasma membrane of the endothelial cell (stippled area fig 1b) was measured and compared with the linear extent of the surface (line *A* fig 1b)

In the third series the percentage of the narrow intercellular space occluded by tight junctions was estimated on a set of micrographs with a final magnification of 56 000–84 000 × The tight junctions at the margins of bile canaliculi were not included in these estimations

No attempt was made to relate these linear measurements to the surface of the intact cell because of the complexity and lack of uniformity of the hepatic cell surface

In the final series the thickness of different segments of the hepatic plasma membrane was measured by a method similar to that described by Millington (64) Electron micrographs taken at an initial magnification of 40 000 × were enlarged photographically to 300 000 × and the membrane was measured with a handlens containing a scale calibrated to 0 1 mm To minimize errors arising from the effects of fresnel fringes all micrographs used for these measurements were taken by one microscopist

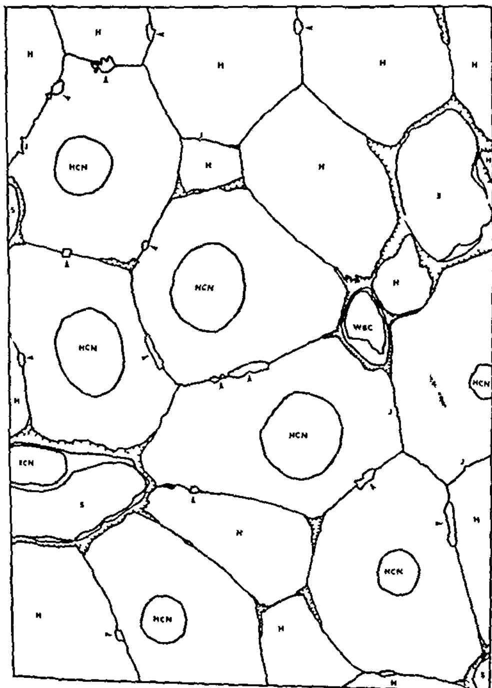
### OBSERVATIONS

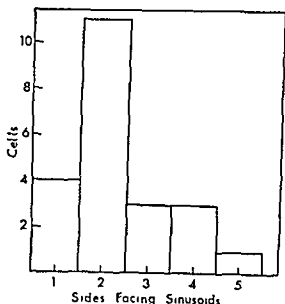
Mouse hepatic cells sectioned through the center of their nuclei appear polygonal in outline (fig 2) A typical hepatic cell has four sides facing its neighbors across a narrow intercellular space and two sides facing the space of Disse surrounding visible sinusoids (figs 2 3) The narrow intercellular space is continuous with the space of Disse and its extensions between adjacent hepatic cells but is interrupted at intervals by bile canaliculi (figs 2–4)

The wall of a bile canaliculus is generally formed by two hepatic cells although as many as one quarter are limited by three cells Each canaliculus formed by two cells is roughly elliptical in outline and its long axis which coincides with a line joining the luminal extremities of the tight junctions at its margins is often of the order of 1 μ in length The long axes of canaliculi comprise about 6% of the perimeter of hepatic cells (table 1) but because of the presence of microvilli the length of plasma membrane within the canaliculi represents a considerably higher percentage of the total plasma membrane of the cell For example in one series of measurements the length of the plasma membrane within each canaliculus was 7 8 ± 0 4 times greater than the long axis of the elliptical cross section of the canaliculus Although occasionally a canaliculus and part of the space of Disse are located in close proximity they are usually separated by a segment of narrow intercellular space several microns in length This intercellular space is always separated from the lumen of the canaliculus by a tight junction (Farquhar and Palade 63) The constituents of junctional complexes at the margins of canaliculi will be considered in more detail in a subsequent section

The space of Disse surrounds each sinusoid following its general contour but it also extends for considerable distances between hepatic cells adjacent to the sinusoid (figs 8 15 16) These extensions of

Fig 2 Tracing of the principle features in a montage of electron micrographs of part of a mouse hepatic lobule The plane of section did not pass through the nuclei of most hepatic cells but did pass through the approximate center of the nuclei (*HCN*) of three cells near the middle of the picture These cells each have six sides of which either one or two contact the space of Disse (stippled) surrounding visible sinusoids (*S*) Most of the remaining sides are in contact along part of their length with extensions of the space of Disse These are shown as stippled areas between adjacent hepatic cells The space of Disse is continuous with the narrow intercellular space which is interrupted at intervals by bile canaliculi (arrowed) tight junctions and desmosomes (not shown) but is generally continuous around studlike junctions (*J*) A white blood cell in the space of Disse (*WBC*) and a nucleus of a sinusoid lining cell (*ECN*) are also shown


H
HCN
S
J
WBC
ECN



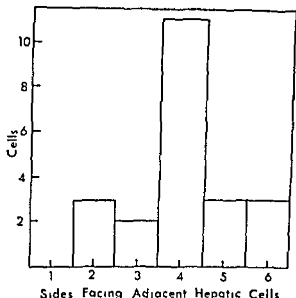
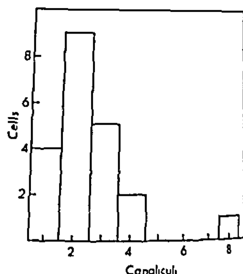
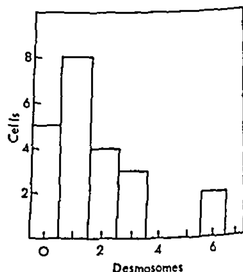




Fig 3 Number of sides of each hepatic cell which face sinusoids (top) and adjacent hepatic cells (bottom) Measurements were made on 22 cells sectioned through the center of the nucleus



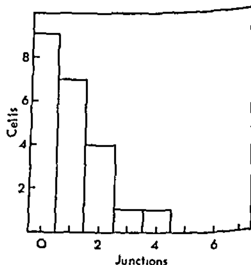




Fig 4 Number of bile canaliculi (top) desmosomes (middle) and studlike junctions (bottom) at the perimeter of *each of* 22 cells sectioned through the center of the nucleus

the space of Disse often appear in sections as isolated dilations of the intercellular space which may bear superficial resemblance to bile canaliculi (fig 9) The criteria used for distinguishing them will be discussed later Although about one third of the sides of hepatic cells face the space of Disse surrounding visible sinusoids (fig 3) more than 80% of the remaining sides contact at least one extension of the space of Disse and about 10% are in contact with an extension of the space of Disse along their entire

TABLE 1

*xtracellular spaces in contact with the perimeter of hepatic cells*

| | | |
|---|---|---|
| verage total perimeter [1] | 76 | ±3 4 μ |
| Percentage of perimeter facing | | |
| Bile canaliculi [2] | 6 3 | ±1 3% |
| Space of Disse [3,4] | 17 | ±2 1% |
| Extension of space of Disse [4] | 25 | ±2 2% |
| Narrow intercellular space | 54 | ±4 5% |

[1] Mean perim ter f 22 ells (from four mi e) sec oned th gh the appro imat enter of the nuclei
[2] Long a of can liculi me ur d between luminal ctrem ti of t ght junctions t th ir margins (i e A fig 1 )
Those portion of the surf c facing pac of i se surrounding i ible sinusoid
Lin extent f cell surf ce measur d a os ases of microvilli (i line "A fig 1b)

ngth In fact an average of more than 0% of the total linear extent of the per meter of each hepatic cell is in contact th a space of Disse or its extensions table 1) Because of the presence of microvilli in this region however a higher percentage of the total length of the plasma membrane is in contact with the pace of Disse In one series of measurements the length of plasma membrane overing microvilli and intermicrovillous spaces was 6 0 ± 0 8 times greater than the linear extent of the cell surface border ing this space

*Surfaces bordering bile canaliculi* The microvilli in bile canaliculi project in more or less random directions from the surface of the hepatic cells and those which are not oriented parallel to the plane of section appear as isolated profiles in the canalicular lumen (figs 9–11) The microvilli contain fine filaments which are more numerous near the limiting membrane and which appear continuous with a cytoplasmic feltwork of fine filaments which surrounds each canaliculus (figs 9–11) We did not observe the more complex structure of microvilli described by David ( 61) in canaliculi and by McNabb and Sanborn ( 64) Graney ( 64) and Rostgaard and Barrnett ( 65) in the intestinal mucosa

Vesicular invaginations were absent from the limiting membranes of canaliculi and no vesicles were present in the adjacent cytoplasm These observations suggest that pinocytosis is not a prominent phenomenon at this surface but they provide no information about the mechanism by which bile constituents leave hepatic cells

In sections that have been fixed in osmium tetroxide and stained with uranium and lead salts the plasma membrane limiting the canaliculi and the space of Disse is thicker than that along the narrow intercellular space (table 2) These membranes all appear slightly thicker in Epon embedded than in Araldite-embedded tissues In all cases the inner and outer leaflets stain with similar intensity (fig 10) and all of the three leaflets are of approximately equal thickness (20–30 A)

The outer dense leaflets of the plasma membranes of adjacent hepatic cells fuse to form the central leaflet of a tight junction at each lateral margin of a canaliculus (figs 9–11) These tight junctions which vary in length from 0 1–0 3 μ (0 16 ± 0 01 μ) are often continuous with intermediate junctions (Farquhar and Palade 63) These intermediate junctions are much less clearly defined than tight junctions and vary in length from 0 1–0 25 μ They differ from tight junctions in that the apposed plasma membranes do not fuse but are separated by a space 130–220 A in width The cytoplasm adjacent to tight junctions and intermediate junctions is relatively dense especially in tissues fixed in osmium tetroxide and it contains a feltwork of fine filaments continuous with that which surrounds each canaliculus (figs 10 11) Intermediate junctions occur only near the bile canaliculi and are not present at any other points on the cell perimeter At the distal end of an intermediate junction a desmosome or a second tight junction is occasionally present (figs 9–11) but in most cases the space between the plasma membranes is continuous with the narrow intercellular space

*Surfaces bordering the space of Disse* The microvilli which extend in various directions from this surface of the hepatic cell are curved and occasionally branched and in thin sections many appear as isolated profiles in the space of Disse (figs 6 8 12–14) These microvilli are of relatively uniform width (0 07–0 12 μ) but are often slightly constricted near their base (figs 12 14) They do not contain either vesicles or surface invaginations and do not appear to have any internal structure apart from a fine filamentous feltwork

TABLE 2

*Thickness of the plasma membrane of hepatic cells*[1]

| Cell | Embedding medium | Location of membrane | | |
|---|---|---|---|---|
| | | Microvilli of bile canaliculi | Microvilli in space of Disse | Facing narrow inter cellular space |
| | | Å | Å | Å |
| 1 | Araldite | 73 | 68 | 63 |
| 2 | Araldite | 75 | 70 | 66 |
| 3 | Araldite | 76 | 79 | 64 |
| 4 | Araldite | 79 | 72 | 66 |
| 5 | Epon | 88 | 82 | 75 |
| 6 | Epon | 88 | 80 | 66 |

[1] Means of ten measurements made along each of three segments of the plasma membrane are shown for six cells These cells were from specimens fixed in osmium tetroxide and stained with both uranium and lead salts In addition the specimens containing cells 1 and 2 were treated with potassium permanganate during dehydration

(figs 13 14) This feltwork which ex tends into the microvilli from the adjacent cytoplasm can often be seen more clearly in specimens fixed in glutaraldehyde and post fixed in osmium tetroxide than in specimens fixed in buffered osmium te troxide alone The ends of almost all the microvilli in our preparations were free in the space of Disse and no microvilli were closely attached to sinusoidal lining cells in the manner described by Wasser man (58)

A finely fibrillar material extends throughout the space of Disse and is con tinuous with the plasma through gaps in the walls of the sinusoids The fibrillar material in the space of Disse has a simi lar density and texture to that in the lu men of the sinusoids (figs 6 12) and is thought to represent the image of fixed plasma protein Small fat droplets 0 1– 0 15 μ in diameter may occur in the space of Disse and in the lumen of the sinusoids (figs 12 15 16) but their concentration is often higher in the space of Disse than within the sinusoids

Two types of vesicular invaginations of the plasma membrane occur between the bases of the microvilli which project into the space of Disse The first type the coated invagination is about 0 1 μ wide and may be sharply constricted at its open ing into the space of Disse (figs 13 14) These invaginations contain material of the same density and structure as that in the space of Disse but do not appear to incorporate fat droplets They are thought to represent stages in the formation of coated vesicles which occur in the hepatic cell cytoplasm near the space of Disse (fig 13) Coated vesicles are also about 0 1 μ in diameter and are limited by a membrane of the same density and thick ness as the plasma membrane Each coated vesicle and coated invagination is bordered by a narrow band of increased cytoplasmic density which contains peri odic dense striations oriented at right angles to the limiting membrane (figs 13 14) Coated vesicles do not ap pear to contain fat droplets but are filled with a fine filamentous material of slightly greater density than that in the space of Disse Vesicles of this type have been de scribed previously in a number of tissues including the rat liver and have been vari ously termed coated complex and al veolate vesicles (Farquhar Wissig and Palade 61 Gray 61 Roth and Porter 62 64 Palay 63 Rosenbluth and Wis sig 63 Bruni and Porter 65)

The second type of invagination is also about 0 1 μ wide and may be constricted at its opening into the space of Disse (figs 12 15 16) Pinocytotic vesicles limited by a membrane which appears identical to the plasma membrane occur in the adja cent cytoplasm (fig 12) and are thought to be derived from these invaginations Pinocytotic vesicles and invaginations dif fer from the coated structures in that the cytoplasmic leaflet of the limiting membrane lacks any form of special ization They contain a finely filamentous material but fat droplets which com pletely fill some vesicles may also be

esent (fig 12) Pinocytotic vesicles are nerally larger and more irregular than ated vesicles and vary in diameter from out 0 1–0 5 μ

A third type of vesicle the small dense esicle is also present in the hepatic cell toplasm near the space of Disse and is ore obvious in specimens fixed in glu araldehyde (fig 15) These vesicles are ll about 0 1 μ in diameter and are filled ith a moderately dense homogenous ma rial They do not resemble either of the ypes of surface invaginations described bove but may contain fat droplets that ould be derived from the space of Disse

In addition to these three types of vesi les large (up to 0 5 μ) and relatively ir egular vesicles" may occur in the hepatic ell cytoplasm very near the space of Disse n regions where the plasma membrane is ortuous (fig 8) These "vesicles" which are limited by a membrane identical to the plasma membrane contain material which appears the same as that in the space of Disse They are thought to represent cross sections of indentations of the space of Disse into the surface of the hepatic cell We did not observe the "granule-contain ing vacuoles with a dense matrix" which were described by Bruni and Porter (65) in the cytoplasm near the space of Disse in rat liver

Although extensions of the space of Disse may bear superficial resemblance to bile canaliculi they differ in a number of morphologic characteristics For instance a finely fibrillar material extends through out the space of Disse but canaliculi are relatively empty except for sparse finely fibrillar or granular material (figs 9–11) The lumen of each canaliculus is always separated from the adjacent narrow inter cellular space by tight junctions but tight junctions do not generally occur at the confluence of the space of Disse with the narrow intercellular space (figs 8–11 15) In sections from the livers of mice which have received exogenous ferritin ferritin molecules are present throughout the space of Disse but are absent from bile canaliculi (see later section) In addition vesicles and surface invaginations occur along the borders of the space of Disse (figs 12–16) but are absent from the borders of canaliculi

*Surfaces bordering the narrow inter cellular space* A narrow intercellular space contacts a large proportion of the perimeter of hepatic cells (table 1) This space is relatively uniform in width (220 ± 14 A) and the limiting membranes which are thinner than those covering other segments of the cell surface (table 2) are generally smooth or slightly curved (figs 5 7 8 17) They may be more convoluted in localized regions and may even separate to form small dilatations of the space These dilated segments often contain small droplets of fat (figs 17 18)

The plasma membranes of the apposed cells are modified at various points to form junctions across the narrow inter cellular space The outer leaflets of the apposed membranes often fuse to form the central leaflet of a tight junction (figs 9 18 19) These central leaflets are al ways thinner than either of the outer leaf lets from which they are derived and may be barely visible even at high magnifica tion (figs 18 19) The tight junctions along the narrow intercellular space re semble those at the margins of bile can aliculi but the adjacent cytoplasm does not contain any evidence of filaments or of increased density (fig 19) The tight junctions are generally straight (fig 18) but irregular and tortuous froms may be present In some cases a series of tight junctions may occur in which the individ ual junctions are separated by short seg ments of narrow intercellular space The distance between the cytoplasmic margins of tight junctions remote from bile cana liculi is 100–150 A and the junctions vary in length from 0 5–2 0 μ (1 0 ± 0 1 μ) They occupy about one fifth (20 ± 2 0%) of the length of cell sides which border the narrow intercellular space

Desmosomes which vary in length from 0 1–0 2 μ (0 13 ± 0 01 μ) may also occur along the narrow intercellular space (fig 11) The structure of hepatic desmosomes will not be considered as they have already been described in detail in the literature (Farquhar and Palade 63)

Studlike processes that project from the surface of one hepatic cell into a concavity in the surface of an adjacent cell (Faw cett 55) are the least common form of junction In this junction the apposed

plasma membranes are of the same thickness and staining intensity as the membranes that border the adjacent narrow intercellular space (figs 17, 19) This space is generally continuous around each studlike process (fig 19) but occasionally the apposed plasma membranes fuse to form a tight junction within the studlike junction

Compound vesicular structures may occur in the hepatic cell cytoplasm near the narrow intercellular space and these are thought to represent transverse sections of studlike junctions (fig 5) The structures are 0 2–0 4 μ in diameter and contain a membrane bounded core which has the same density and texture as the surrounding cytoplasm This core is separated from the external limiting membrane by a narrow (100–200 A) ring of low density which presumably represents a segment of narrow intercellular space within the studlike junction Both the external membrane and the membrane surrounding the core have the same thickness and structure as the plasma membrane bordering the narrow intercellular space

The desmosomes studlike junctions and tight junctions remote from bile canaliculi are distributed more or less at random along the narrow intercellular space A few cell sides lack cell junctions except for those at the margins of the canaliculi and an occasional cell side lacks both cell junctions and bile canaliculi

*Coated and pinocytotic* vesicles and invaginations occur in the region of the narrow intercellular space but are much less common than near the space of Disse Neither small dense vesicles nor vesicular structures believed to be cross sections of pockets of the space of Disse have been observed in the hepatic cell cytoplasm near the narrow intercellular space

*Distribution of exogenous ferritin* A high concentration of ferritin molecules was present in the narrow intercellular space the space of Disse and the hepatic sinusoids of all mice which had received injections of ferritin (figs 12 13 15 16 18 19) We were not able to determine whether consistent variations existed in the concentration of ferritin molecules between these areas

At the margins of bile canaliculi ferritin molecules were occasionally present between the apposed plasma membranes of intermediate junctions but did not appear either between the leaflets of tight junctions or in the lumens of canaliculi Ferritin molecules were not found between the leaflets of tight junctions elsewhere on the plasma membrane although they did appear within short segments of narrow intercellular space separating adjacent tight junctions (fig 18) From these results we conclude that tight junctions are impermeable to ferritin and that the narrow intercellular space is continuous around circumscribed segments of the tight junctions remote from bile canaliculi Ferritin molecules were present between the apposed plasma membranes within studlike junctions (fig 19), verifying the continuity of the narrow intercellular space around these junctions

Ferritin molecules were also present within coated and pinocytotic invaginations and vesicles in the region of the space of Disse and the narrow intercellular space (figs 12 13) Some of the pinocytotic vesicles contained both fat droplets and ferritin molecules (fig 12) The concentration of ferritin molecules in the vesicular structures which are thought to represent cross sections of small pockets of the space of Disse was the same as the concentration in the adjacent areas of the space of Disse

## DISCUSSION

In conventional histologic sections of mammalian liver the lobule seems to consist of more or less straight cords one parenchymal cell in thickness that converge from the periphery of the lobule to terminate at the central vein After analysing such preparations Elias (49) concluded that the radial cords of parenchymal cells in fact represent sections through relatively flat plates of cells These plates are separated from one another by sinusoids of relatively uniform diameter which converge towards the central vein into which they ultimately empty

When thin sections of liver are examined with the electron microscope most sinusoids appear circular in outline The appearance of the sinusoids in thin sec

...ons can best be reconciled with their appearance in the considerably thicker conventional histologic sections if we assume that they pursue a relatively serpentine course through fenestrations in the plates of liver cells Their lateral digressions may not be sufficiently great to carry them out of the plane of a thick histologic section but could cause them to repeatedly leave and re-enter the plane of thin sections

If the sinusoids do pursue a serpentine course we should expect that the plane of thin sections would sometimes transect the space of Disse surrounding a sinusoid which was out of the plane of section (fig 3) These tangential sections of the space may resemble transverse sections of radial extensions of the space of Disse Thus some of the "extensions of the space of Disse" which we observed may have represented tangential sections through the roughly annular space surrounding the sinusoids However most appeared to be sectioned normally and are thought to represent radial extensions of the space of Disse between hepatic cells adjacent to the sinusoids

*The extracellular spaces* Bile canaliculi extend throughout the liver lobule but are separated from the remainder of the extracellular spaces by tight junctions Although Rouiller ( 56) and Ashworth and Sanders ( 60) described open connections between the canalicular lumen and the space of Disse a number of authors have provided evidence to the contrary (Fawcett 55 Hampton 58 Novikoff and Essner 60 Daems 61 Farquhar and Palade 63) Farquhar and Palade ( 63) demonstrated that tight junctions exist at the margins of canaliculi and concluded that these tight junctions are impermeable to protein molecules In our studies *tight junctions* were always present at each canalicular margin and these appeared to prevent the entry of ferritin molecules into the canaliculi Farquhar and Palade ( 63) also provided evidence which suggests that tight junctions may be impermeable to smaller molecules and possibly to water If this is true all the biliary constituents from the blood must pass through hepatic cells in order to reach the canaliculi

The narrow intercellular space was wider in our studies than in those of other authors (Fawcett 55 Ashworth and Sanders 60 Daems 61) but these variations may reflect different methods of specimen preparation (c f Hampton 60) Major changes in the width of the space may result from relatively minor changes in the volume of the adjacent cells during fixation and embedding The width of the narrow intercellular space may also very depending on the species (c f David 61) and on the physiological condition of the animal For example the width of the space between epithelial cells in the intestine apparently varies in different nutritional conditions (Sjostrand 63)

Several types of modifications of the cell surfaces occur along the narrow intercellular spaces In tissues embedded in methacrylate Fawcett ( 55) and Daems ( 61) described stud like processes which extend from one hepatic cell into a corresponding recess on the surface of an adjacent cell However when Hampton ( 60) examined liver specimens embedded in Araldite he was unable to demonstrate these processes and concluded that they were alterations due to distortion during embedding or sectioning We have found these processes in specimens embedded both in Araldite and in Epon and believe that they probably do occur in the living animal However because they are relatively uncommon and because the narrow intercellular space is continuous around the processes we believe that they do not exert any significant function in cell cohesion

Desmosomes are present along the narrow intercellular space but the apposed plasma membranes do not appear to be joined by any type of cement substance and the narrow intercellular space appears to continue without interruption between the two membranes The apposed plasma membranes with their associated fibril containing areas of increased cytoplasmic density may act as focal points of structural rigidity However as desmosomes are not common in the liver lobule and as each desmosome covers a very small proportion of the total cell surface their importance is probably slight

It seems likely that the main intercellular attachment devices in the liver lobule are the tight junctions which occur at the margins of the canaliculi and at frequent intervals along the narrow intercellular space These tight junctions appear to be essentially similar to the external compound membrane of Robertson (58) and the quintuple layered cell interconnections of Karrer (60) Those which occur along the narrow intercellular space remote from canaliculi are not constant in position with reference to any landmark of the hepatic cell and they are not present along all the cell sides which face the narrow intercellular space For this reason we believe that these junctions do not form a belt of any length around the cells but that they are relatively circumscribed areas of fusion of the plasma membranes The conclusion is supported by the results of experiments in which ferritin molecules introduced into the circulation were found along the length of the narrow intercellular space and between adjacent tight junctions Although it is possible that ferritin molecules could have reached some parts of the narrow intercellular space by vesicular transport through hepatic cells the amount of ferritin present in vesicles in these experiments did not appear sufficient to warrant serious consideration of this alternative explanation

*Transport of materials between hepatic cells and extracellular spaces* It has been suggested that some substances which are removed from the blood by cells lining the sinusoids are subsequently transferred to hepatic cells (Wasserman 58) However we were unable to find either microvilli closely attached to lining cells or any evidence to suggest that exogenous ferritin molecules could be transferred directly from the lining cells to microvilli of hepatic cells These observations concur with those of Hampton (58) and seem to indicate that plasma constituents must enter the space of Disse before they can be absorbed by hepatic cells

Coated and pinocytotic vesicles are both concerned with uptake of these plasma constituents by hepatic cells but show different degrees of selectivity in the nature of the materials they will ingest Our observations on the uptake of exogenous ferritin molecules by coated vesicles would seem to confirm that the role of coated vesicles in protein uptake in the liver resembles that in other tissues (Farquhar Wissig and Palade 61 Rosenbluth and Wissig 63 Roth and Porter 64 Bruni and Porter, 65) However coated vesicles in liver cells do not appear to concentrate ferritin preferentially as they do in the cells of toad spinal ganglia (Rosenbluth and Wissig 63)

Pinocytotic vesicles also incorporate ferritin molecules but these vesicles are predominantly concerned with the uptake of fat droplets from the space of Disse and the narrow intercellular space Fat droplets have been described previously in both the space of Disse and adjacent cytoplasmic vesicles but their mode of entry into the hepatic cells has remained obscure (c f Ashworth Steinbridge and Sanders 60 French 63) Our observations appear to confirm the suggestion of Trotter (60) that pinocytotic invaginations and vesicles play an important part in this process However we were unable to confirm the observation of Schlesinger and Essner (65) that coated invaginations and vesicles may incorporate fat droplets into hepatic cells

The fat droplets in our preparations were all between 0 1 μ and 0 14 μ in diameter and we were unable to determine whether they were chylomicrons synthesized in the gut wall from exogenous fat or lipoproteins derived from endogenous sources The very low density lipoproteins vary in size up to 0 14 μ and closely resemble small chylomicrons (c f Courtice and Garlick 62 French 63) In addition physiologic experiments have revealed that both chylomicrons and lipoproteins are removed from the blood in the liver (French and Morris 58 Bragdon and Gordon 58 Stein and Shapiro 60 Rodbell Scow and Chernick 64) If the fat droplets present in our preparations were chylomicrons their uniform small size *may indicate that only smaller chylomi*crons can pass through the gaps in the sinusoidal walls It is also possible that some of the droplets may have been lipoproteins which had been discharged from hepatic cells and were passing through the space of Disse to the bloodstream

Although microvilli cover a large propor n of the surface of hepatic cells their le in the transfer of materials between e hepatic cell and the extracellular aces is unknown Vesicles and surface vaginations do not occur in microvilli it appear to be confined to the inter icrovillous spaces In this respect micro- lli of hepatic cells resemble those of epi elial cells of the intestinal wall (c f alay and Karlin 59 Lacy and Taylor 2) However fat droplets may be ab orbed into intestinal microvilli (Rost aard and Barrnett 65) but do not appear o enter microvilli of hepatic cells

*Structure of the plasma membrane* On the basis of studies of tissues fixed in otassium permanganate Robertson (59 61) postulated that cellular membranes onsist of a central lipid leaflet bordered on both surfaces by layers of nonlipid ma erial Subsequent studies have revealed hat considerable variations may occur within the framework of this simple unit membrane model In tissues fixed in os mium tetroxide the structure of plasma membranes may vary between different cells and between different parts of the same cell (Wissig 62 Sjostrand 63 Farquhar and Palade 63 Millington 64) For instance the plasma membrane cover ing the microvilli of intestinal epithelial cells is substantially thicker than that covering the lateral and basal surfaces of the cell (Sjostrand 63 Millington 64) in the intestine These differences may indi cate that variations in the physiologic characteristics of the cell are reflected in slight changes in the structure of the membranes

Variations in the methods used during preparation of specimens for examination may also cause alterations in the structure of membranes In many epithelial cells which have been stained with either ura nium or lead salts the outer leaflet of the plasma membrane is thinner and less dense than the inner leaflet If the cells are treated with potassium permanganate during dehydration or if they are stained with both uranium and lead salts the outer leaflet can be visualized more readily (Farquhar and Palade 63) In our study no differences were apparent between the inner and outer leaflets of the plasma membranes of hepatic cells stained with both uranium and lead salts

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*Abbreviations*

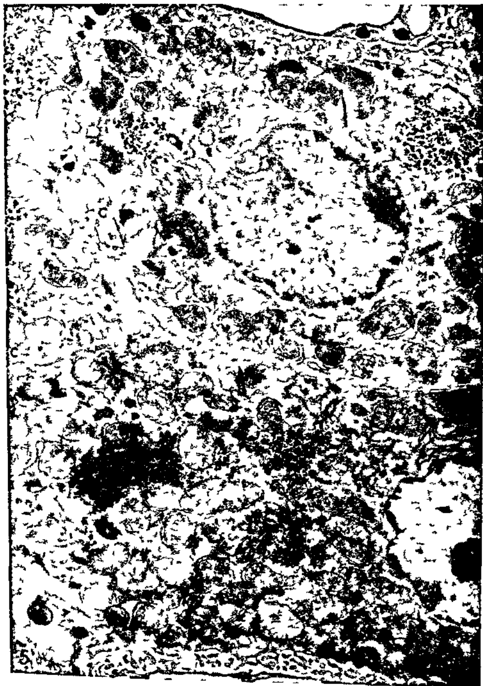
| | |
|---|---|
| *c* bile canaliculus | *e* extension of the space of Disse |
| *d* space of Disse | *f* fine filaments |
| *dv* dense vesicle | *z* zonula occludens (tight junction) |

The mice from which the specimens shown in figures 8 12 13 15 16 18 and 19 were obtained received an intraperitoneal or intravenous injection of ferritin solution prior to sacrifice

PLATE 1

EXPLANATION OF FIGURE

5 Hepatic cells of the mouse liver are shown at relatively low magnification The borders of sinusoids appear at the upper and lower margins of the figure The hepatic cells are roughly polygonal in outline Two bile canaliculi (*c*) interrupt the narrow space between hepatic cells A cross section of a studlike junction (arrow) is shown near the center of the figure × 10 000


C
C
5

## PLATE 2

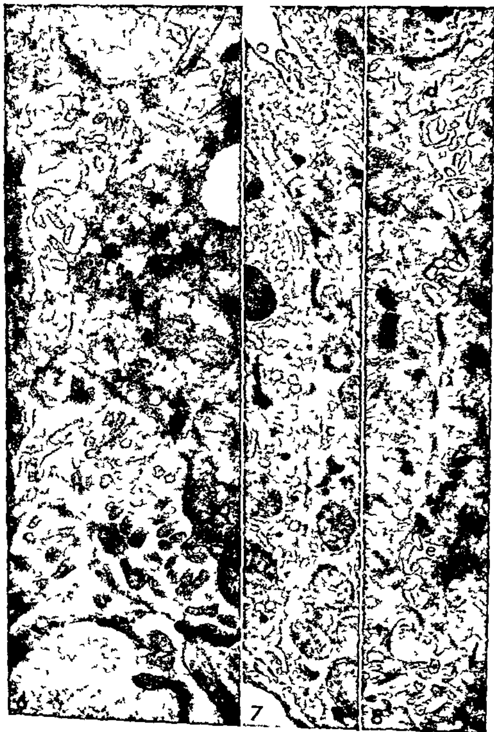
### EXPLANATION OF FIGURES

The figures illustrate the diversity of structure of intercellular spaces between hepatic cells The intercellular space extends between spaces of Disse situated at the top and bottom of each figure

6 The widely separated surfaces of the hepatic cells are coated with microvilli and the space between them is broadly continuous with the spaces of Disse at the top and bottom of the figure The lining cells of both sinusoids are sectioned obliquely This field probably contains a single sinusoid sectioned at a point where it makes a serpentine bend In the center of the figure the sinusoid passes out of the plane of section which intercepts only its bordering space of Disse × 27 000

7 A narrow space separates the hepatic cells in this field A bile canaliculus (*c*) with cross sections of microvilli filling its lumen interrupts the space midway along its length A zonula occludens poorly visualized at this low magnification occurs at each lateral margin of the canaliculus × 15 000

8 A bile canaliculus (*c*) is situated close to a space of Disse (*d*) in the narrow space between hepatic cells Its lumen filled with microvillous profiles is separated from the space of Disse above and the narrow intercellular space below by zonulae occludentes A lumen (*e*) containing a few microvillous profiles dilates the narrow space near the sinusoid at the lower border of the figure It is identified as an extension of the space of Disse because ferritin molecules can be detected in its lumen in the original micrograph and it lacks zonulae occludentes in its lateral margins (see text) A vesicular structure (arrow) believed to represent a cross section of an invagination of the space of Disse is present in the lower part of the figure × 25 000


7
8
d
e

## PLATE 3

### EXPLANATION OF FIGURE

9 A bile canaliculus in a specimen fixed in buffered osmium tetroxide solution The numerous microvillous profiles in its lumen are limited by distinct unit membranes and sparse filamentous or granular material is seen in their cores Fine filaments (*f*) appear in the bordering cytoplasmic matrix Zonulae occludentes (*z*) occur at the lateral margins of the canaliculus and are bordered by fine filaments A short distance from one zonula occludens the narrow intercellular space is obliterated (arrow) where the plasma membranes of the hepatic cells are fused together The lumen of the canaliculus appears empty and there is no sign of vesicles either forming or emptying along its border × 88 000


Z
Z

## PLATE 4

### EXPLANATION OF FIGURES

10 A zonula occludens (z) bordered by fine filaments is situated at a *lateral margin of a bile canaliculus* *Below it the plasma membranes* are sectioned obliquely and their relationship to each other cannot be discerned There are abundant fine filaments in the adjacent cytoplasm The membranes again appear sectioned perpendicularly near the lower margin figure (arrow) and here they are fused together × 144 000

11 At the lateral margin of a bile canaliculus the plasma membranes of adjacent hepatic cells fuse to form a zonula occludens (z) near its lumen A desmosome (macula adhaerens) can be recognized in the lower portion of the figure Between these two elements of a junctional complex the plasma membranes are sectioned obliquely for most of their length but they can be seen to be clearly separated at two points (arrows) It is not possible to decide with certainty whether an intermediate junction (zonula adhaerens) occurs in this region A mesh of fine filaments stretching from the zonula occludens to the desmosome lines the cytoplasmic surface of the plasma membranes × 120 000


Z
Z
10

## PLATE 4

### EXPLANATION OF FIGURES

10 A zonula occludens (z) bordered by fine filaments is situated at a lateral margin of a bile canaliculus Below it the plasma membranes are sectioned obliquely and their relationship to each other cannot be discerned There are abundant fine filaments in the adjacent cytoplasm The membranes again appear sectioned perpendicularly near the lower margin figure (arrow) and here they are fused together × 144 000

11 At the lateral margin of a bile canaliculus the plasma membranes of adjacent hepatic cells fuse to form a zonula occludens (-) near its lumen A desmosome (macula adhaerens) can be recognized in the lower portion of the figure Between these two elements of a junctional complex the plasma membranes are sectioned obliquely for most of their length but they can be seen to be clearly separated at two points (arrows) It is not possible to decide with certainty whether an intermediate junction (zonula adhaerens) occurs in this region A mesh of fine filaments stretching from the zonula occludens to the desmosome lines the cytoplasmic surface of the plasma membranes × 120 000


1
2
12

## PLATE 5

### EXPLANATION OF FIGURE

12 A dense concentration of ferritin molecules is seen in the sinusoidal lumen in the upper portion of the figure Small droplets of moderate density (arrows) within it are identified as either chylomicrons or very low density lipoprotein particles Several vesicles in the lining cell bordering the lumen contain numerous ferritin molecules A few contain in addition a dense homogenous substance Tortuous microvilli of the subjacent hepatic cell extend into the space of Disse which also contains a dense concentration of ferritin The surface of the hepatic cell is deeply invaginated at one point (*1*) between the bases of adjacent microvilli A pinocytotic vesicle containing ferritin molecules and faintly outlined lipid particles (2) appears free in the cytoplasm near the surface of the hepatic cell × 91 000

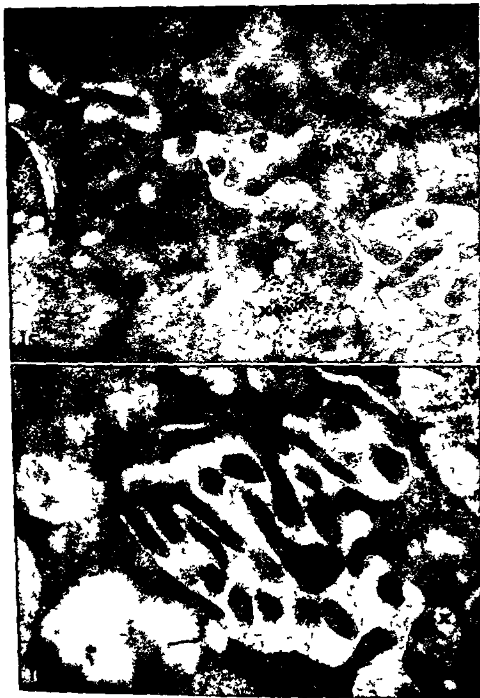

dv
13
dv

## PLATE 6

### EXPLANATION OF FIGURES

13 Arrows indicate coated vesicles containing ferritin in the sinusoidal lining cell (upper part of the figure) and hepatic cell (lower part of the figure) A vesicle containing only homogenous dense material (*dv*) appears near the surface of the hepatic cell × 67 000

14 Two coated invaginations (arrows) appear along the surface of a hepatic cell A vesicle with dense homogenous content (*dv*) appears to the left of one of the invaginations × 128 000



## PLATE 7

### EXPLANATION OF FIGURES

15–16 Both figures show portions of the space of Disse that extend some distance away from a sinusoid and dilate the space between hepatic cells Numerous microvilli protrude into the extension of the space which contains many ferritin molecules and spherical particles presumably lipid which are not easily visualized because of their lack of density The cytoplasm bordering the extensions contains numerous small dense vesicles (*x*) with a content that matches in diameter and density the spherical particles in the lumen Some of the vesicles also contain ferritin Two of them (arrows) are seen opening at the surface of hepatic cells × 48 000 (fig 15) × 62 000 (fig 16)


19

## PLATE 8

### EXPLANATION OF FIGURES

17 Two studlike intercellular junctions appear in the upper part of the figure The upper one is cut in cross section the lower one in longitudinal section Within both junctions the hepatic cells are separated by a distinct intercellular space In the lower part of the figure a cluster of lipid droplets are seen in a localized dilation of the narrow intercellular space × 102 000

18 At the top margin and in the center of the figure the plasma membranes of neighboring hepatic cells are fused together obliterating the narrow intercellular space Between the zones of fusion a distinct narrow space containing numerous ferritin molecules is present × 122 000

19 Studlike junctions appear in the central and lower portions of the figure Within each junction the adjacent hepatic cells are separated by a distinct space which contains ferritin The plasma membranes of the hepatic cells are fused together in the lower part of the field × 111 000


19

# n the Occurrence of a Fibrous Lamina on the Inner spect of the Nuclear Envelope in Certain ells of Vertebrates[1]

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ABSTRACT The fine structure of the *fibrous lamina* on the inner aspect of the nuclear envelope in cells of various invertebrates is reviewed and attention is drawn to the common occurrence of a similar but thinner layer in nuclei of cells in verte brates Certain superficial similarities are pointed out in the relations of *fibrous lamina* to the nuclear pores and of the *basement lamina* to the endothelial capillary pores It is suggested that in future considerations of the physiological exchange of materials between nucleoplasm and cytoplasm the properties of the fibrous lamina must be taken into account

A complex supporting layer of fine fila ıents closely applied to the inner aspect f the nuclear envelope has been described ır *Amoeba proteus* (Pappas 56 Mercer ›9) *Gregarina melanopli* (Beams et al ›7) and the neurones of the ventral nerve ord of *Hirudo medicinalis* (Gray and uillery 63 Coggeshall and Fawcett 64) 'his structure has been called the fibrous ımina No corresponding layer appears o have been described as yet for nuclei of ertebrate cells Since the introduction of ıldehyde fixatives for electron microscopy and the widespread practice of staining ıltra thin sections with lead and uranyl ons the inner leaf of the nuclear envelope ıow often appears to be thicker than the outer However at high magnification the nembrane itself is found to have the usual limensions its appearance of greater thick ıess being due to a continuous layer of losely compacted filamentous material applied to its inner surface In view of the increased interest in the permeability proprties of the nuclear envelope and the mechanisms of nucleocytoplasmic inter action (Baud 65 Lowenstein and Kanno 63 65) it would seem timely to draw attention to the occurrence of such a layer in a number of cell types in vertebrates and to suggest that it is the counterpart of the *fibrous lamina* already described for the nuclei of certain Protozoa Gregarines and Annelids Although the exact nature and functional significance of this layer cannot yet be specified it must be taken into account in future considerations of the physiological properties of the nuclear envelope

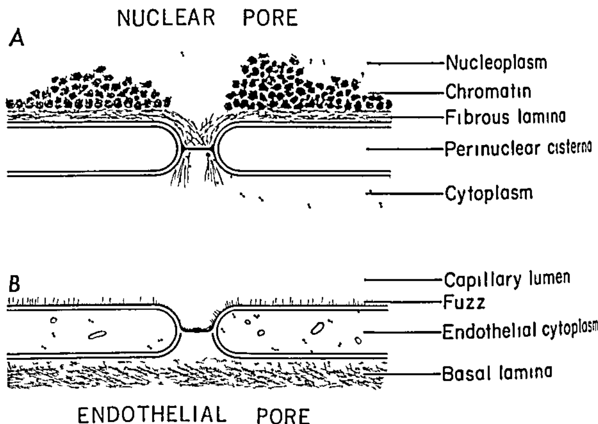
## MATERIALS AND METHODS

The observations recorded here were incidental to investigations on the ventral nerve cord of the leech the germinal epithelium and interstitial tissue of the cat testis the guinea pig ductus epididymidis and the intestinal mucosa of the Congo eel The fixation was accomplished in collidine buffered 1 3% osmium tetroxide in the case of the leech material The vertebrate tissues were fixed in collidine buffered 5 5% glutaraldehyde followed by 1% osmium in the same buffer Embedding was in Epon and the sections were stained with uranyl acetate and lead citrate The micrographs were made on RCA EMU 3G electron microscope

## OBSERVATIONS

To facilitate comparison of the nuclear envelope of an invertebrate with that of vertebrates a micrograph of a ganglion cell nucleus from the leech is presented in figure 1 A prominent fibrous lamina (Gray and Guillery 63 Coggeshall and Fawcett 64) is found on the inner aspect of the nuclear envelope In diametric sec tions it presents a scalloped profile with thin regions alternating with thicker re

[1] Supported in part by grants GM-6729 and GM 10182 from th Division of General M di l Sciences Nation l In titute f H alth United State Public H lth Service




Text figure 1 Diagrams comparing the topographical relation of structures associated with a pore in the nuclear envelope (A) with the arrangement of components at a pore in a capillary endothelial cell (B) Both are traversed by a thin septum the pore diaphragm and by a layer of filamentous material — the fibrous lamina of the nuclear envelope in the one case and the endothelial basal lamina ( basement membrane ) in the other

gions that project inward to form ridges or septa outlining a system of cylindrical compartments open at one end to the nucleoplasm and closed at the other by the nuclear envelope In subtangential sections (fig 1 inset) the fibrous lamina presents a honeycomb appearance of dense septa delimiting circular areas of nucleoplasm of lower density At high magnification the septa are observed to be composed of fine filaments 30 to 50 A in diameter (see at arrows inset to fig 1) In the nuclear envelope at the bottom of each concavity in the fibrous lamina there is a typical nuclear pore The filamentous material is very thin if not entirely lacking over the pore opening

After glutaraldehyde and osmium fixation the chromatin in the nucleus of mammalian smooth muscle cells is disposed in masses around the periphery and is preserved in the form of granules that stain intensely with the combination of uranyl and lead ions The closely packed granular subunits of chromatin form a dark beaded row that runs parallel to but is not in contact with the smooth contour of the inner leaf of the nuclear envelope (fig 2) Instead the chromatin seems to be separated from the dense line representing the membrane proper by a layer 150 to 200 A thick consisting of a very fine textured material of lower density than either the membrane or the chromatin This layer therefore appears as a thin lighter zone of uniform thickness interposed between the dense chromatin and the innermost unit membrane of the bilaminar nuclear envelope (*b* in the inset to fig 2) Owing to its thinness and its compact organization the filamentous nature of this layer has not been convincingly demonstrated in the vertebrate material illustrated here Nevertheless it seems permissible by analogy to assume that its ultimate structural components are basically similar to those of

corresponding layer in the nucleus of tain invertebrates and to refer to it as *fibrous lamina*

This layer lining the nuclear envelope of Schwann cells of peripheral nerve (fig and endothelial cells (fig 8) is very ular in thickness and density to that of ooth muscle (figs 2 and 4) In the estinal epithelium of the Congo eel the rous lamina is thicker and stains more avily and therefore appears as a con icuous dark layer (fig 3) Because it arly matches the chromatin and the nu ar membrane in density its precise nits are more difficult to define

In the nucleus of the interstitial cells of e cat testis the sparse heterochromatin curs in small clumps scattered through it the nucleoplasm and in a thin inter pted layer against the nuclear envelope ig 7) Even at low magnification the ner element of the nuclear envelope ap ars unusually thick (see at arrows fig ) At higher magnification this dark line found to be made up of the inner leaf of e nuclear envelope and a heavily stained *brous* lamina of very uniform thickness fig 9)

In the nucleus of leech neurones as escribed above there is invariably a cir ular *discontinuity* in the fibrous lamina ver *each* pore in the nuclear envelope It as anticipated that the same would be rue in vertebrate nuclei and that where he central portion of a pore was included n the section the fibrous lamina would ap ear interrupted or greatly attenuated This *expectation* is not borne out The ibrous layer often appears to continue un hanged *across the* pore whether the sec ion passes through the pore eccentrically or through the center For example in fig ure 9 and the inset to figure 8 the sharp ness of the image of *the pore diaphragms* and surrounding membranes would indi cate that the *sections* pass through the center of the pores in a plane normal to the membranes *Yet in these* figures the fibrous lamina clearly continues across the pore and has a sharply *defined* inner mar gin (see at arrows figs 8 and 9) In other cell types the inner edge *of the* fibrous lamina is poorly defined over the pore (figs 2 and 5) Nevertheless it contains material of appreciable density on both sides of its diaphragm This appearance suggests that in these instances the mate rial of the fibrous lamina is present at the pores but more loosely organized there than it is elsewhere along the nuclear envelope

The nucleus of the cat spermatocyte in cluded here for comparison is representa tive of a number of cell types in which a continuous fibrous lamina cannot be de tected Its chromatin is dispersed and no dense clumps are present around the rim of the nucleus to provide the discontinuity in contrast that facilitates visualization of the fibrous lamina Other small granular components of the nucleoplasm are scat tered along the nuclear envelope however and it is quite evident that instead of being held away by an intervening layer these granules are often in direct contact with the inner membrane (fig 6) At the pores a moderately dense substance is found on either side of the pore diaphragm (see at arrow fig 6) This probably corresponds to the structure referred to by other authors as "the annulus" It is suggested that the substance of the annulus is the same as that comprising the fibrous lamina If this is so it would seem that in some cells it forms a continuous layer lining the nuclear envelope while in others such as the spermatocyte it is lacking except at the pores

## DISCUSSION

In previous reports of a fibrous lamina in *Amoeba proteus* and in *Hirudo medicinalis* the suggestion was offered that this struc ture might have a supporting function providing a fibrous internal reinforcement for the nuclear envelope in cells with un usually large nuclei This interpretation relating the fibrous lamina to nuclear size is difficult to defend however for among invertebrates there are many examples of cells with enormous nuclei in which this layer is not developed Moreover in amoebae other than *Amoeba proteus* the fibrous lamina seems to be lacking even though the nuclei may be of comparable size The published descriptions of the fibrous lamina in the leech were based upon examination of the very large packet glial and ganglion cells of the ventral nerve cord but it is also present in the endothelial

of nuclear pores is the same as that which in some cells forms a continuous lining for the nuclear envelope then further characterization of the pore substance will be facilitated by selection of cell types for study which have a well developed fibrous lamina

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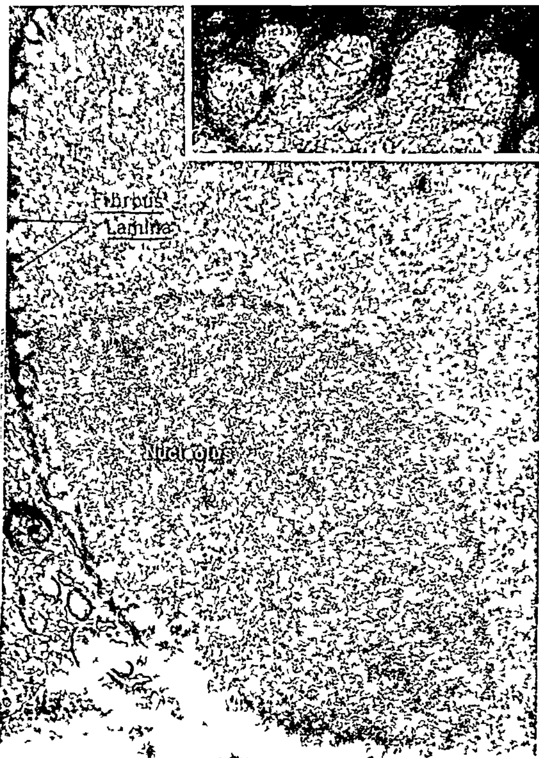
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*Footnote added in proof* Since submission of this paper it has come to my attention that Dr Gianfranco Patrizi in an abstract in the Proceedings of the Eighth International Congress of Anatomists Wiesbaden 1965 described a similar dense layer on the inner aspect of the nuclear envelope to which he applied the term *zonula limitans nucleum* This layer was reported to be of consistent occurrence in some cell types but absent in others Among those in which it was found are Schwann cells endothelial cells fibroblasts kidney epithelium and amniotic and HeLa cells in tissue culture

## PLATE 1

### EXPLANATION OF FIGURE

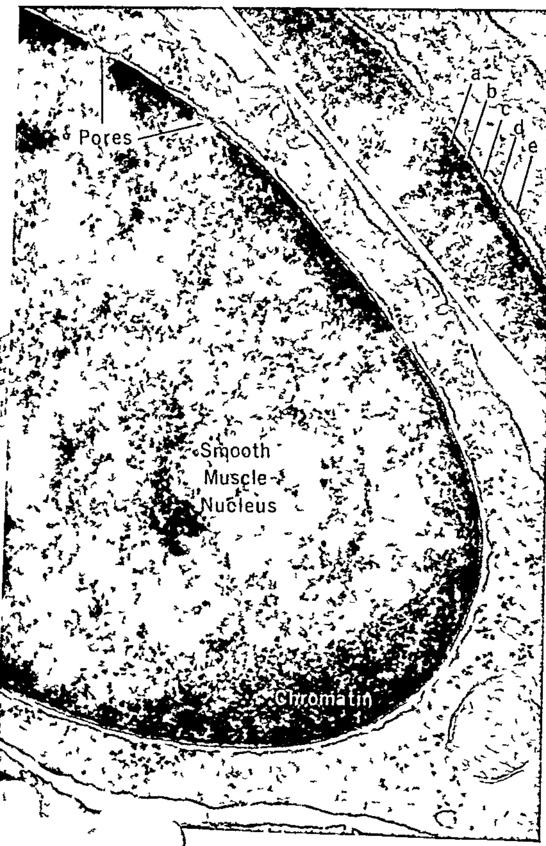
1 An electron micrograph of a sector of the nucleus in a ganglion cell from the ventral nerve cord of the leech *Hirudo medicinalis* The lower half of the figure is dominated by a large nucleolus consisting mainly of ribonucleoprotein granules The nucleolus is held away from the nuclear envelope by a thick fibrous lamina whose alternate thick and thin regions give the margin of the nucleoplasm a scalloped appearance Thickenings of the fibrous lamina project inward to form a hexagonal pattern of septa that delimit shallow cylindrical compartments of nucleoplasm A nuclear pore is situated at the bottom of each compartment where the fibrous lamina is thinnest In subtangential section the fibrous lamina presents the honeycomb configuration illustrated in the inset The arrows indicate areas where the component filaments are resolved (Micrograph by Dr Richard Coggeshall )


Fibrous
Lamina
Nucleolus

## PLATE 2

### EXPLANATION OF FIGURE

2 A portion of a smooth muscle cell from the guinea pig epedidymis The heavily stained nuclear chromatin is disposed around the periphery but does not appear to be in direct contact with the inner membrane of the bilaminar nuclear envelope The thin layer of material of lower density intervening between the chromatin and the inner leaf of the nuclear membrane is believed to correspond to the fibrous lamina of the leech nucleus At higher magnification in the inset the chromatin is indicated at *a* the fibrous lamina at *b* the inner membrane of the nuclear envelope c the perinuclear cisterna *d* the outer membrane of the nuclear envelope at *e*


Pores
a
b
c
d
e
Smooth
Muscle
Nucleus
Chromatin

## PLATE 3

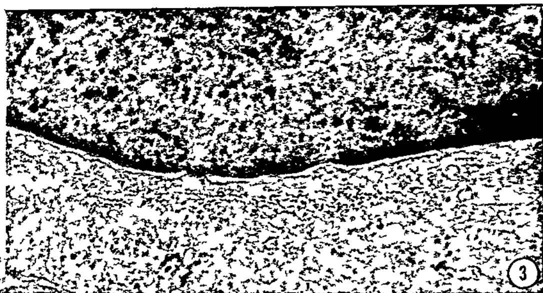
### EXPLANATION OF FIGURES

3 A small area of the nucleus and adjacent cytoplasm from an intestinal epithelial cell of *Amphiuma tridactylum* In this case the fibrous lamina has stained more heavily and appears as a moderately dense fine textured layer about 300 A thick The fibrous lamina appears to continue across the nuclear pore but is somewhat thinner and less dense in this region

4–5 Additional examples from smooth muscle and Schwann cell nuclei showing the fibrous lamina as a thin layer of lower density interposed between the dense chromatin and the dark line representing the inner membrane of the nuclear envelope At the arrows a fairly well demarcated inner margin of the fibrous lamina can be followed across the pore region In the other pore without an arrow the material of the fibrous lamina appears less dense and has poorly defined limits

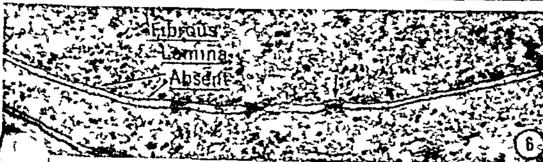
6 A portion of the nucleus (above) and adjacent cytoplasm (below) from a cat spermatocyte In contrast to the other examples on this plate a continuous fibrous lamina is absent Granular components of the nucleoplasm are clearly in direct contact with the inner membrane of the nuclear envelope There is however the usual moderately dense material associated with both sides of the diaphragm at the nuclear pore (at the arrow)

IF wc tt




Chromatin
Fibrous
Lamina
Nuclear
Isterna
Inner & Outer
Membranes

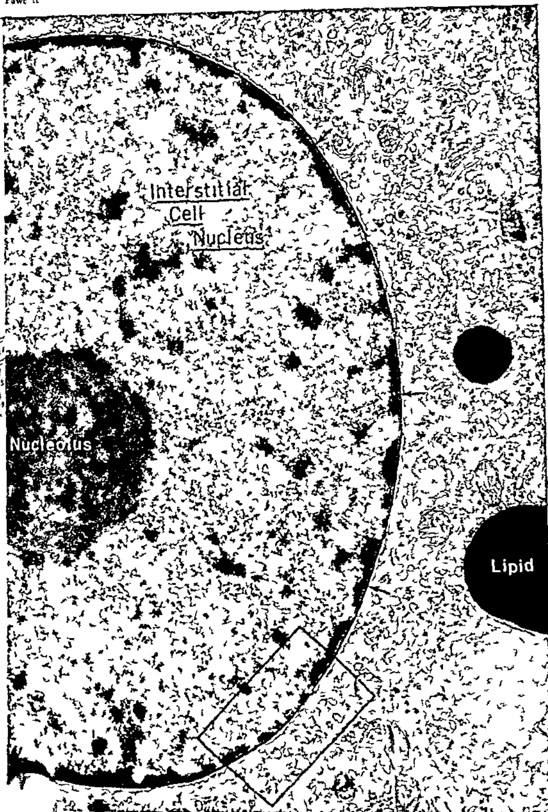



Fibrous
Lamina
Absent

## PLATE 4

### EXPLANATION OF FIGURE

7 Nucleus of cat interstitial cell showing a thin peripheral accumulation of chromatin interrupted at the sites of nuclear pores At this low magnification at the arrows the inner member of the bilaminar nuclear envelope appears to be several times the thickness of the membrane on the cytoplasmic surface When an area such as that enclosed in the rectangle is examined at higher magnification (fig 9) it is found that this appearance of greater thickness is due to the presence of a fibrous lamina closely applied to the nuclear surface of a membrane of the usual thickness (80–90 A)

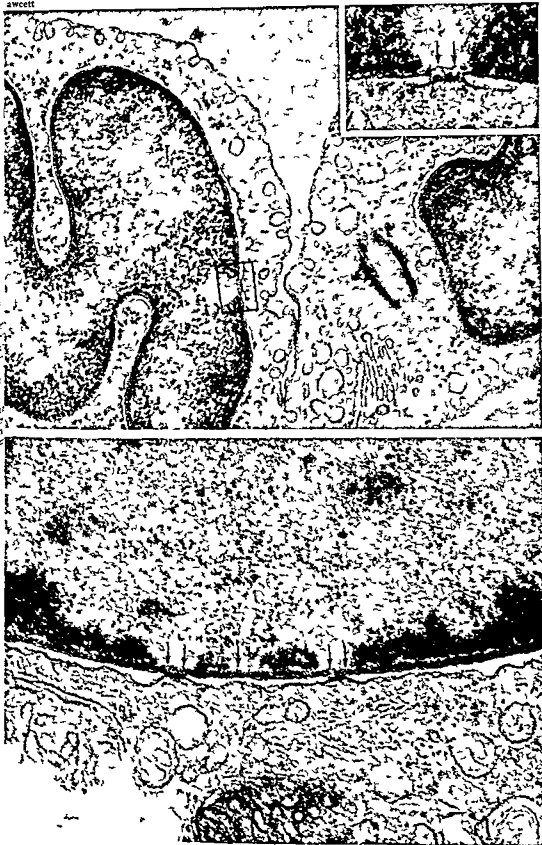

Interstitial
Cell
Nucleus
Nucleolus
Lipid

## PLATE 5

### EXPLANATION OF FIGURES

8 Two neighboring endothelial cells of a blood vessel in the interstitium of the cat testis As in the smooth muscle and Schwann cells in figures 2 4 and 5 the dense chromatin granules at the periphery of the nucleus are aligned in a row parallel to the nuclear envelope separated from it by a narrow zone of lower density representing the fibrous lamina The region of a nuclear pore enclosed in the rectangle is shown at higher magnification in the inset The arrows point to the sharp line of demarcation of the fibrous lamina over the pore and the area of low density in the adjacent nucleoplasm

9 A portion of an interstitial cell nucleus similar to that enclosed in the rectangle on figure 7 The fibrous lamina is more heavily stained than in most of the other figures and has a texture suggesting that it is a feltwork of randomly oriented thin filaments The arrows point to the sharp inner margin of the fibrous lamina which appears to continue across the pores



## PLATE 5

### EXPLANATION OF FIGURES

8 Two neighboring endothelial cells of a blood vessel in the interstitium of the cat testis As in the smooth muscle and Schwann cells in figures 2 4 and 5 the dense chromatin granules at the periphery of the nucleus are aligned in a row parallel to the nuclear envelope separated from it by a narrow zone of lower density representing the fibrous lamina The region of a nuclear pore enclosed in the rectangle is shown at higher magnification in the inset The arrows point to the sharp line of demarcation of the fibrous lamina over the pore and the area of low density in the adjacent nucleoplasm

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# lectron Microscopic Study of the Gingivo-dental ınction of Man [1]

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*ABSTRACT* Erupted teeth with attached gingival tissue were obtained from 15 patients ranging in age from 4 to 26 years After preliminary glutaraldehyde fixation the area to be studied was excised with a high speed dental drill The tissue was decalcified in Versene subdivided into smaller blocks and post fixed in 2% buffered osmic acid fixative Epon embedded sections were stained with uranyl acetate and lead citrate prior to being examined in the electron microscope

The results indicate that (1) an attachment is present between the epithelial cuff and enamel or cementum (2) the attachment apparatus of epithelium to tooth structure consists of hemidesmosomes and a cementing layer resembling the base ment lamina of various epithelium-connective tissue junctions (3) the attachment may be to two types of cuticle of undetermined origin or directly to the tooth and (4) apical to the epithelial attachment the gingiva is connected to cementum by parallel bundles of collagen fibrils which extend from the gingiva into the cementum The cementum itself consists of parallel collagen fibrils the periodicity of which is in register between adjacent fibrils The fibrils are embedded in a rather sparse granular matrix

In 1929 Orban and Mueller wrote a comprehensive and critical review on the microscopic structure of the gingival crevice area with particular emphasis on the structure of the epithelial attachment Their conclusion at that time confirmed Gottliebs previous findings (21) that an epithelial attachment can be found on enamel and that contrary to previous beliefs the attachment was not located at the cemento-enamel junction only (Black 15) This concept remained more or less unchallenged until 1952 when Waerhaug published a monograph in which he denied the existence of an organic epithelial attachment to enamel and provided some experimental proof that the bottom of the normal gingival crevice was situated at the cemento-enamel junction He visualized the epithelium adjacent to the tooth as a tightly adapted cuff which could however be displaced from the tooth by the introduction into the potential space of thin metallic strips Since that time numerous articles have been written to support Gottliebs concept of an organic attachment (Becks '29 Kronfeld 30 Toller '39 Baume 52 Baume 53 Macapanpan 54 Ussing 55 Orban Bhatia Kollar and Wentz 56 Weinreb 60) as well as Waerhaug's proposal of an epithelial cuff (Greulich 61 Beagrie and Skougaard 62 Skougaard and Beagrie 62 Greulich 62 Trott and Gorenstein 63 Hunt and Paynter 63 Engler Ramfjord and Hiniker 65 McHugh and Zander 65)

Recently Stern (62 63) using electron microscopy described the epithelial attachment in the continuously erupting incisors of rats This attachment appeared to exist both on enamel and cementum However because morphologically and physiologically the dento-gingival relationship in rodent teeth is markedly different from that of primate teeth (Aldritt 61) it still remained to be shown that a true epithelial attachment was present in man

In the present report which is part of a study to develop a satisfactory method for processing periodontal tissues for electron microscopic study evidence will be presented which confirms the existence of an attachment between gingival epithelium and the teeth of man This attachment is morphologically similar to that which is found between epithelial lining membranes and their connective tissue support

[1] This investigation was supported by grant DA 136 from the National Research Council of Canada Associate Committee on Dental Research

ticle A in the remainder of this paper occasionally appeared continuous with though structurally different from the namel matrix (fig 6) Cuticle A fre ently demonstrated appositional lines its structure which was quite dense and anular (fig 7)

Although cuticle A was frequently quite rominent near the cemento-enamel junc on (figs 4 7) it narrowed considerably ronally (fig 8) sometimes ending sud nly (fig 12) or continuing as a thin proximately 100 mμ layer between the ithelial attachment and the enamel ig 9) In one specimen cuticle A was llowed from the apical border of the namel to a point coronal to the epithelial ttachment In this instance the epithelial tachment was to cuticle A The most ronal cells of the attachment were sep rated from the cuticle This separation ccurred between the cuticle and the base ent lamina the latter remaining attached the hemidesmosomes of the attachment pparatus (fig 10)

A second type of cuticle was observed ither covering cuticle A (fig 11) or in irect apposition to enamel (fig 12 15) r cementum (fig 13) It was generally ss granular than cuticle A usually pres nting a mottled appearance (figs 14 15) t was usually found opposite the cells of he epithelial attachment and will be re ferred to as cuticle B Where the epithelial attachment extended onto cementum uticle B continued along the external sur ace of the cementum to end near the pical portion of the epithelial attachment Although cuticle B was generally found as n intervening layer between the epithelial attachment and the tooth surface it was not always necessarily present in that posi tion Thus an epithelial attachment could be found directly to cuticle A (figs 7 9 14) or directly to tooth enamel (fig 16) without any intervening structure resembl ing cuticle B It should also be noted that the cuticle binding bacterial plaque to tooth surface (fig 17) was morphologi cally indistinguishable from cuticle B

The junction between the tooth or the cuticles and the epithelial cells was ac tually mediated by a thinner less dense granular layer similar to the basement lamina of epithelium The basement la mina was always found between the epi thelial cells and the adjacent tooth or cuticles (figs 8 to 15) It varied in width from 400 to 1 200 Å Together with the hemidesmosomes present along the por tion of the plasma membrane facing the tooth it appeared to constitute the actual attachment apparatus of the epithelial cells to the teeth Occasionally invagina tions of the cell membrane adjacent to the basement lamina were observed which were filled with a substance morphologi cally identical to that of the basement lamina (figs 11 12)

Circular profiles were occasionally found within the basement lamina of the attach ment apparatus (figs 12 13 15) These probably correspond to microvilli from nearby epithelial cells They frequently demonstrated a series of concentric layers on their periphery which dimensionally corresponded to the layers observed in hemidesmosomes These layers have re cently been described in detail by Stern ( 65) in the hemidesmosomes of gingival epithelium The reader should refer to this paper for further detail The appear ance of the limiting membrane of these microvilli suggests the existence of belt like or cup-like hemidesmosomes com pletely surrounding the tip of these cyto- plasmic extensions

*The connective tissue attachment*

The epithelial membrane forming the epithelial attachment was connected to the gingival connective tissue by means of an attachment apparatus consisting of hemidesmosomes and a basement lamina similar to that found in other connective tissue-epithelial junctions (figs 7 18) Collagen fibrils from the connective tissue came in contact with but never crossed the basement lamina

Apical to the epithelial attachment the collagen fibrils of the gingival connective tissue became oriented into parallel bun dles which were inserted into the cemen tum and became an important component of the organic matrix of cementum (figs 5 19 20) The other component of the organic matrix appeared as a dense often granular substance which surrounded the collagen fibrils within the cementum This interfibrillar granular material was most

## METHOD AND MATERIALS

Teeth which visually and radiographically appeared free of periodontal disease were obtained at the time of extraction from 15 patients ranging in age from 4 to 26 years and immediately placed in ice cold buffered glutaraldehyde fixative Concentrations of glutaraldehyde of 5% and 6 25% were used Buffers which were adjusted to pH 7 4 included phosphate cacodylate veronal acetate and s collidine

Some teeth were obtained with an intact lingual or facial covering of oral mucosa or bone and oral mucosa Separation of the covering tissues from the adjacent tissues prior to extraction was achieved by the use of a sharp scalpel and when needed a mallet and chisel Other teeth were obtained by extraction without regard for the preservation of an intact relationship between the teeth and surround soft tissues Even though elevators which mechanically separate the tooth from the soft tissue were used in some of these extractions some soft tissue invariably remained attached to the extracted tooth especially in the cervical region

After an interval of 1 to 3 hours of preliminary glutaraldehyde fixation the teeth were trimmed with a no 56 fissure bur in a dental engine equipped with an air driven turbine under a water spray coolant so that only a very thin layer of mineralized dental tissue remained in contact with the surrounding soft tissue The trimmed specimen was left to fix overnight in the glutaraldehyde fixative It was then transferred to a 0 25 M solution of Versene [2] made up in the appropriate buffer or in the glutaraldehyde fixative itself Demineralization generally required 3 to 7 days After demineralization was completed the specimens were subdivided into smaller blocks approximately 1 mm thick These were post fixed for 1 to 3 hours in 2% osmic acid fixative buffered to pH 7 4 dehydrated in graded aqueous solutions of ethanol and embedded in Epon (Luft 61) A total of 130 blocks were examined in this study The preparation of the tissues for embedding is diagrammatically illustrated in figure 1 Sections were cut at 0 1 μ on a Porter Blum MT 2 ultramicrotome collected on either bare or carbon reinforced formvar coated grids and stained with a saturated solution of uranyl acetate in 50% ethanol and with lead citrate (Reynolds 63) Sections were examined in a Philips EM 200 microscope at 60 or 80 kv using the double condenser and a 20 μ objective aperture

## RESULTS

In all teeth examined whether extracted with care so as to not disturb the gingival attachment or whether extracted in routine fashion evidence of a firm connection of soft tissue with tooth was always found In routinely extracted teeth examination of the region between the remaining soft tissue and the tooth indicated that the actual junction between soft and hard tissue was frequently intact but tears were evident within the epithelium or connective tissue portion of the attachment (figs 2 3)

The examination of teeth erupted for as long as 20 years still revealed what appeared as a firm connection of soft tissue to enamel No major differences in the appearance of the attachment could be attributed to the type of tooth the age or sex of the patient

In the remainder of this paper the term apical refers to the location of a structure closer to the root tip of the tooth The term coronal signifies that the location is closer to the crown end of the tooth

### *The epithelial attachment*

Because the epithelial attachment of the tooth was frequently connected to cuticular structures covering the tooth rather than directly to enamel or cementum it is first necessary to describe these cuticular structures

In cases where the cementum did not overlap enamel the cervical portion of the enamel was often covered with an electron dense layer which ended at the most apical border of the enamel flush with the cementum (fig 4) Cementum was sometimes found over the enamel apical to the epithelial attachment In such cases the dense layer continued uninterrupted between the enamel and the cementum to the most apical border of the enamel (fig 5) This dense layer will be referred to as

[2] Ethylene diamine tetra acetic acid Beresworth Chemical Co Framingham Mass U S A

The material examined in this study in ided only fully erupted teeth with the ginal attachment consisting of reduced amel epithelium having presumably en partially or completely replaced by attachment consisting of cells derived om the oral epithelium and outer layers the reduced enamel epithelium The mness of the attachment was evident ice despite the mechanical stress to hich some of our material had been subcted during routine extractions the at chment of epithelial cells to the tooth mained intact in some areas When tears d occur they generally appeared within e epithelium itself The finding of a rm attachment to the tooth and tears ithin the epithelium must however be iterpreted with care in view of the pre arative procedure The latter included emineralization with a chelating agent despite the fact that tissues were first fixed n glutaraldehyde it is possible that re noval of calcium ions by chelation might esult in loss of cellular adhesiveness nith consequent tears within the epithe lium without affecting the attachment of hese cells to the tooth or its cuticles (Conan 61 Berwick and Coman 62) Thus he tears within the epithelium may not necessarily be due to mechanical stress but ather might reflect a sudden weakening of the desmosomes due to calcium deple on during the preparative procedure

Of particular interest is the finding that the attachment apparatus consisting of hemidesmosomes and the basement la mina which connects the epithelium to the tooth or its cuticles is remarkably similar to that seen at the junction of any epi thelial lining with its underlying connec tive tissue The basement lamina of most epithelial membranes including the gin giva consists of a dense homogeneous layer ~ 400 A wide separated from the epithelial plasma membrane and its hemi desmosomes by a well defined less dense layer of ~ 400 A (Listgarten 64 Mel cher 65 Stern 65) The basement la mina of teeth does not reveal as clearly these double strata of different density Also it is wider (~ 400–1 200 A) and of less constant width than the basement la mina of other epithelial membranes How ever the average width (~ 800 A) of the basement lamina of the epithelial attach ment closely approximates the combined width of the two layers of the basement lamina of other epithelial membranes

Of additional interest is the fact that such an attachment apparatus can exist between epithelium and a tissue such as enamel which is not of mesenchymal origin The finding adds further weight to the recent observations by Midgley and Pierce ( 63) Pierce Midgley and Sri Ram ( 63) Pierce Beals Sri Ram and Midgley ( 64) and Mukerjee Sri Ram and Pierce ( 65) that at least some basement mem branes are of epithelial origin The appear ance in this study of structures resembling secretory vacuoles along the epithelial cell membrane facing the tooth suggests that cells of the epithelial attachment may be involved in some secretory activity The fact that the contents of these vacuoles structurally resemble the substance of the cementing layer lends support to the pos sibility that the epithelial cells may be active in the synthesis of this layer

The resemblance of the epithelial attach ment to epithelial-connective tissue junc tions does not end with the morphological appearance It is well known that if an epidermal surface is locally denuded of epithelium the adjacent epithelial cells can quickly glide over the denuded con nective tissue and cover the defect Yet despite their relatively rapid motion along a connective tissue surface it is extremely difficult to separate the epithelial cells from the underlying connective tissue by means of a force applied perpendicularly to the epidermal surface

A similar situation probably exists with respect to the epithelial attachment which is firmly resistant to forces which might tend to detach the epithelium from the tooth yet cells in contact with the tooth can quickly migrate from the basal por tions of the epithelial attachment toward the gingival crevice (Greulich 61 Beagrie and Skougaard 62 Skougaard and Be agrie 62 Greulich 62 Hunt and Paynter 63) The theoretical aspects of some of the mechanisms which may play a part in the attachment of epithelial cells to tooth enamel have been recently reviewed by Schultz Haudt and co-workers ( 63) and the reader is advised to consult that pub-

clearly observed as localized deposits near to the cementum surface (fig 22) generally closely related to cytoplasmic extensions from adjacent connective tissue cells presumably cementoblasts

In the coronal portion of cementum near the cemento enamel junction collagen fibrils were sometimes loosely arranged with a relatively large proportion of interfibrillar material (fig 4) More apically, the fibrils became denser and were arranged in remarkably parallel bundles both before and after entering the cementum (figs 20 21) The packing of these fibrils was so precise that the multiple bands which constitute the ~ 600 A period of collagen fibrils could be followed over large areas of cementum as a series of lines roughly parallel to the cementum surface (fig 21) No evidence of spur like cementum formations were evident at points on the cementum where collagen fibrils entered from the adjacent connective tissue

The bundles of collagen fibrils extended in a more or less straight line from the cemental surface to the dentino-cemental junction In general no marked curving of the fibrils was observed within the cementum Within 1 to 3 μ from the dentino cemental junction the cemental fibrils appeared to lose their closely packed parallel arrangement (fig 23) In this region the bands of adjacent fibrils were not as perfectly in register as the bands of fibrils closer to the surface There was no apparent connection between the collagen fibrils of the cementum and dentine although the fibrils of one tissue were closely interdigitated with the fibrils of the other The junction between dentine and cementum was easily identified because in this location the collagen fibrils of the dentine appeared to lack any kind of orientation in contrast to the more regularly arranged fibrils of cementum No evidence of a basement lamina could be detected at the cemento-dentinal junction Neither was there any evidence that cemental fibers assumed a parallel relationship to the dentinocemental junction as is the case in rat incisors (Stern 64)

Where the epithelial attachment was located on cementum the collagen fibrils within the cementum ended abruptly at the cementum surface A cuticle of B was generally found on the surface between the attachment of the epithelium and the cementum (fig 13)

## DISCUSSION

Although much has been written with respect to the existence of an e... attachment versus an epithelial cuff the two concepts regarding the relation of the epithelium to the tooth are necessarily mutually exclusive Much the recent work in this area points to the fact that the epithelium although tightly bound to the tooth is capable of rapid migration along its surface Recent stud... using autoradiographic techniques ...lich, 61 Beagrie and Skougaard 62, Skougaard and Beagrie 62, Greulich 62, Hunt and Paynter 63 McHugh and Zander 65) have indicated not only that of the epithelial attachment are capable of rapid migration along the tooth but that the whole epithelial attachment probably turns over within a period of as little as five days in mice (Beagrie Skougaard '62) and six days (Skougaard and Beagrie 62) Ramfjord and Hiniker (65) in to confirming the concept of a dynamic epithelial attachment observed that the turnover of cells in the attachment ... slower than in the sulcus epithelium and that the turnover seemed to increase in inflamed areas They interpreted these findings as an indication of decreased sealing action by the epithelial attachment in inflamed areas

The epithelial attachment in monkeys appears to undergo a period of maturation shortly after eruption during which the reduced enamel epithelium its original component is gradually replaced by the apical downgrowth of squamous epithelial cells derived from the oral epithelium and outer layers of the reduced enamel epithelium (McHugh 61 63 Cohen 62 McHugh and Zander 65) McHugh ('63) stated that this replacement requires approximately 18 months in monkeys and is probably longer in man Cohen s experiments indicated that the presence over the enamel of the reduced enamel epithelium is essential for the normal development of the mature attachment

!) Kronfeld ( 28) and Gottlieb and Or ı ('38) are of the order of several hun d micron and are believed to be associ d with increased occlusal stress No nental spurs comparable in size with se seen in rat incisors were observed in s investigation

The results of a study to determine the ure of the attachment found between erupted human teeth and their surround soft tissue has been recently completed e findings to be published shortly indi e that morphologically the attachment reduced enamel is similar to that of the thelial attachment of erupted teeth listgarten 66)

ACKNOWLEDGMENTS

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lication for further details and additional references

No evidence was found to support the concept of a fibrillar attachment as presented by Baume (53) with tonofibrils from the epithelial cells extending through the attachment area to become continuous with the enamel matrix

From the electron micrographs obtained in this study only two morphologically distinct types of cuticles could be identified The first type referred to as cuticle A consisted of an electron dense granular layer which appeared to correspond by definition to a primary enamel cuticle (Gottlieb 21 Orban 26) mainly because of its location over the enamel only and between the enamel and cementum when the latter overlapped the enamel This cuticle was occasionally followed past the epithelial attachment over the erupted portion of the enamel (fig 10)

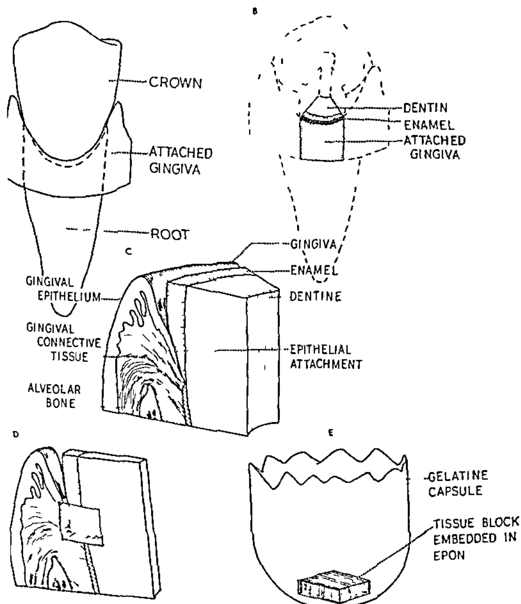
The other type of cuticle referred to as cuticle B was less granular than cuticle A and frequently presented a mottled appearance It was generally found in contact with the epithelial attachment lying directly on enamel (fig 12) or cementum (fig 13) or over cuticle A (fig 11) However there was no evidence to suggest that cuticle B was secreted by epithelial cells Furthermore the cuticle attaching bacterial plaque to the tooth surface (fig 17) appeared morphologically similar and is unlikely to be a secretion product of epithelial cells It is quite possible that cuticle B is derived from organic probably protein components of tissue fluid or saliva

Because demineralized material was employed in this study it was not possible to determine whether either or both types of cuticle were initially mineralized It is of some interest however to note the morphological resemblance of cuticle A to the granular cementum matrix (fig 19) Perhaps it corresponds to a form of coronal cementum devoid of a collagenous fibrillar component The existence of a cementum layer over tooth enamel of human teeth has been recently suggested by Levine Glimcher and Bonar (64) However the coronal cementum described by these authors is morphologically different from cuticle A

The connective tissue attachment of the gingiva to the tooth was mediated by collagen fibrils extending from the gingival connective tissue into the cementum where they formed the so-called Sharpey's fibrils In close proximity to the dentine these fibrils did not assume a parallel arrangement to the dentino-cemental junction Such an arrangement of the collagen fibrils within the cementum had been reported by Selvig (63) and Stern (64) However these authors were working with mouse and rat incisors respectively The difference in arrangements of fibrils within the cementum of continuously erupting teeth and human teeth could be explained simply on the basis of the relatively rapid rate of eruption of the rodent teeth In contrast to cementum from continuously erupting rat incisors the cementum of human teeth contained a much higher concentration of collagen fibrils This is in accord with similar observations by Kuwaza (63) Frank and Nalbandian (63) and Stern (64) The concentration of interfibrillar matrix was correspondingly smaller in human cementum

Some collagen fibrils which were embedded in cementum did not continue into the gingival connective tissue but rather stopped near the cementum surface These were located under areas of new cementum matrix deposition (fig 22) or deep to the epithelial cuticle when the epithelial attachment was present on cementum (fig 13) Thilander (61) has recently described the dissolution of cemental fibrils near the cementum surface in rat molars affected with periodontal disease Whether embedded fibrils become lysed at the cementum surface in disease only or whether such lysis may occur as part of a physiological process of tissue turnover is not clear

Although cemental spurs have been described in rat incisors as well as in human teeth the order of magnitude of these structures is quite different from rat to man The spurs described by Stern (64) in rat incisors are quite frequent along the cementum surface surrounding almost every individual fibril entering the cementum with a conical projection approximately 0 1–0 2 μ in size The spurs described in human cementum by Heuser


CROWN
ATTACHED GINGIVA
ROOT
B
DENTIN
ENAMEL
ATTACHED GINGIVA
C
GINGIVA
ENAMEL
DENTINE
GINGIVAL EPITHELIUM
GINGIVAL CONNECTIVE TISSUE
EPITHELIAL ATTACHMENT
ALVEOLAR BONE
D
E
GELATINE CAPSULE
TISSUE BLOCK EMBEDDED IN EPON

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## PLATE 1

### EXPLANATION OF FIGURES

Fig 1 Diagram illustrating the method used in obtaining and orienting specimens of the cemento-enamel junction area for study with the electron microscope

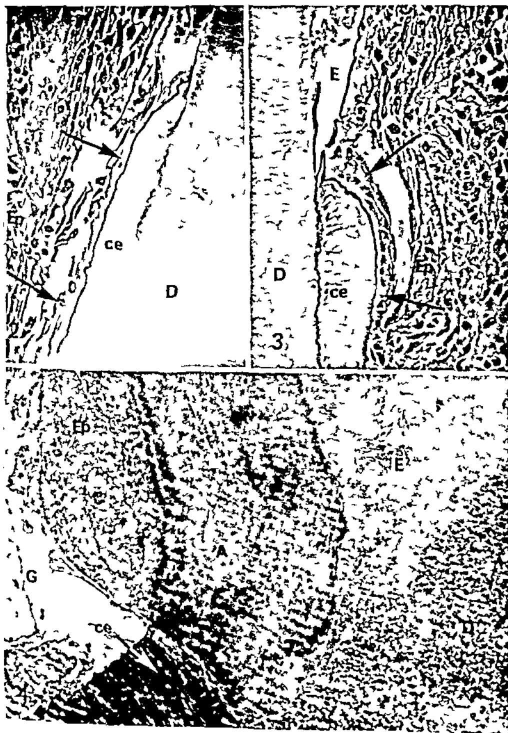
A Tooth with attached gingiva Fixed as shown in gluteraldehyde for 1–3 hours

B Tissue remaining after trimming away of excessive tissue Fixed as shown in gluteraldehyde for approximately 24 hours then demineralized in Versene

C Side view of tissue block shown in B prior to subdivision into smaller blocks

D Smaller blocks are excised under a dissecting microscope after demineralization in Versene The shape of the block facilitates its subsequent orientation in the gelatin capsule

E Properly oriented block in an Epon filled gelatin capsule


Ep
ce
D
E
D
ce
Ep
3
Ep
E
A
G
ce
D

## *Abbreviations*

*A* type A cuticle
*B* type B cuticle
*Ba* bacteria
*bm* basement lamina adjacent to connective tissue
*ce* cementum
*Ceb* cementoblast
*cem* interfibrillar cementum matrix
*CL* basement lamina adjacent to the tooth
*D* dentine
*Dcej* dentino cemental junction
*E* enamel space
*Em* enamel matrix
*Ep* cells of epithelial attachment
*G* gingival connective tissue
*Gc* gingival crevice
*Hd* hemidesmosome
*mv* microvilli
*V* secretory vacuole
*zo* zonula occludens

## PLATE 2

### EXPLANATION OF FIGURES

2–3 Tear within epithelial attachment Note that epithelial cells remain attached to the tooth structure (arrows) Lower first deciduous molar of five year old boy Phase contrast × 400

4 Cemento-enamel junction area Upper second premolar of 15 year old boy × 4 500

E

A

G

ce

5

Em

A

6

## PLATE 3

### EXPLANATION OF FIGURES

5 Cemento-enamel junction area just apical to epithelial attachment Note type A cuticle between enamel space and cementum Lower second deciduous molar of five year old boy × 10 000

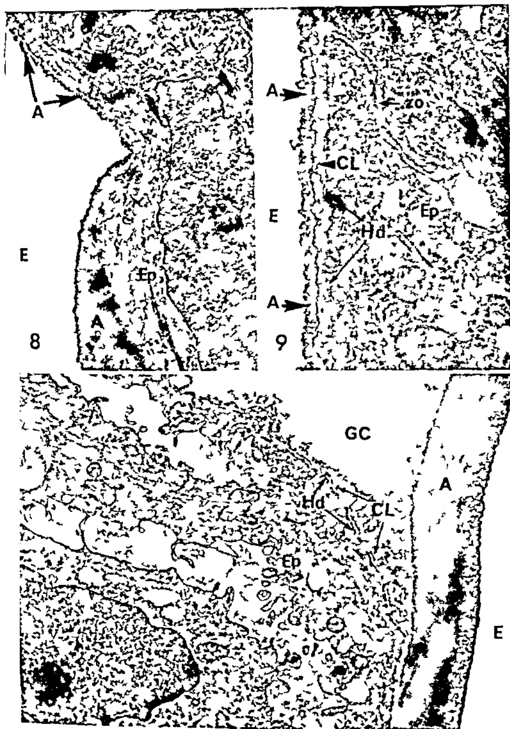
6 Junction of type A cuticle and enamel matrix near cemento-enamel junction Upper second premolar of 15 year old boy × 30 000


E
Em
A
Ep
G

## PLATE 4

### EXPLANATION OF FIGURE

7 Epithelial attachment on type A cuticle just coronal to cemento-enamel junction area shown in figure 4 Note appositional layers within the cuticle Upper second premolar of 15 year old boy ×7 000


A
E
Ep
A
8
A
zo
CL
E
Ep
Hd
A
9
GC
Hd
CL
Ep
A
E

## PLATE 5

### EXPLANATION OF FIGURES

8 Type A cuticle narrowing to ~ 100 mμ layer between enamel and epithelial attachment Upper second premolar of 15 year old boy × 14 000

9 Continuation of type A cuticle coronal to area shown in figure 8 between enamel space and basement lamina of epithelial attachment Upper second premolar of 15 year old boy × 36 000

10 Epithelial attachment to type A cuticle Note that where epithelium is separated from cuticle basement lamina is retained along cell surface opposite to the hemidesmosomes Lower first deciduous molar of five year old boy × 11 000

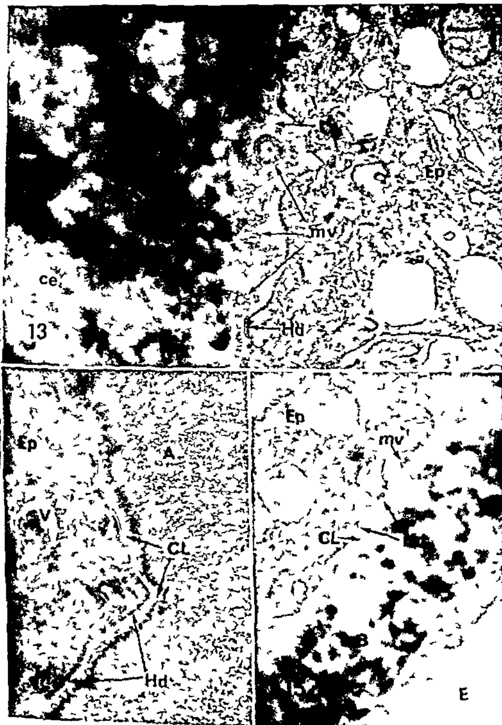

Em
Ep
CL
V
A
B
Hd
11
E
CL
Hd
mv
V
Cn
Ep
12

## PLATE 6

### EXPLANATION OF FIGURES

11 Epithelial attachment to type B cuticle which in turn lies over the type A cuticle covering the enamel Structure resembling a discharg ing vacuole can be seen to contain a granular material similar to that of the basement lamina Lower first deciduous molar of five year old boy × 37 000

12 Section just coronal to figure 11 showing the end of type A cuticle (arrow) The type B cuticle continues coronally in direct apposition to the enamel Lower first deciduous molar of five year old boy × 39 000


Ep
mv
ce
13
Hd
Ep
A
V
CL
Hd
Ep
mv
CL
B
E

## PLATE 7

### EXPLANATION OF FIGURES

13 Epithelial attachment to cementum Note the cemental fibrils ending at the cementum surface (small arrows) which is covered by a type B cuticle The epithelial cell is attached by hemidesmosomes and a basement lamina to the cuticle Cross sections of microvilli may be seen in the basement lamina Peripheral appearance of microvilli is suggestive of cup like or belt like hemidesmosomes Lower first deciduous molar of five year old boy × 50 000

14 Magnified portion of figure 18 demonstrating the attachment of epithelium to a type A cuticle by hemidesmosomes and a basement lamina Upper second premolar of 15 year old boy × 40 000

15 Magnified portion of figure 12 showing the attachment of epithelium to a type B cuticle through hemidesmosomes and a basement lamina Lower first deciduous molar of five year old boy × 45 000

Cl
Hd
Ep
Em
Hd
d
E
B
Ba
17

## PLATE 8

### EXPLANATION OF FIGURES

16 Epithelial attachment to enamel through hemidesmosomes and a basement lamina in the absence of a cuticle Upper lateral incisor of 26 year old man × 58 000

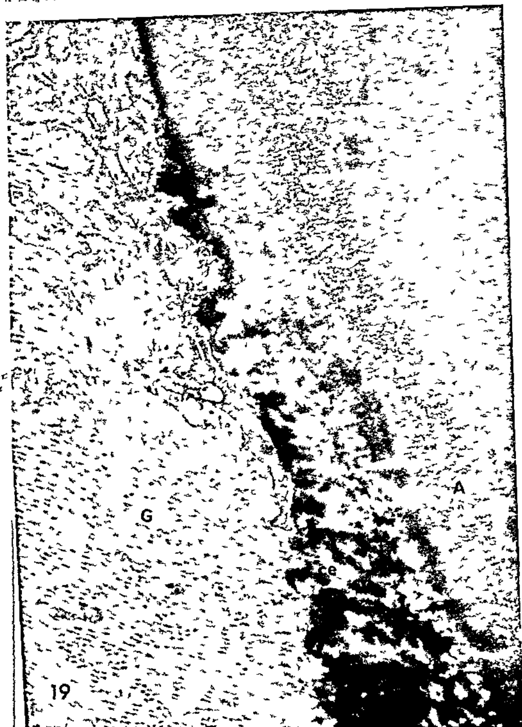
17 Bacterial plaque attached to enamel through a type B cuticle Lower first deciduous molar of five year old boy × 40 000


E
Hd
bm
A
G
CL
18

## PLATE 9

### EXPLANATION OF FIGURE

18 Epithelial attachment to type A cuticle Note degenerative changes in the cells of the epithelial attachment (compare with connective tissue cells which are relatively well preserved) Degenerating epithelial cells may correspond to cells of the reduced enamel epithelium which have not yet been replaced by down growing cuff of oral epithelium Upper second premolar of 15 year old boy × 14 000


A
G
e
19

## PLATE 10

### EXPLANATION OF FIGURE

19 Section just apical to figure 18 showing a cementum layer with embedded collagen fibrils on the surface of a type A cuticle Upper second premolar of 15 year old boy × 19 000

## PLATE 11

### EXPLANATION OF FIGURES

20 Insertion of gingival collagen fibrils into cementum immediately coronal to the alveolar bone margin Note periodic striations of collagen fibrils in register within the cementum Arrows indicate junction of cementum and gingival connective tissue Lower second deciduous molar of five year old boy × 35 000

21 Acellular cementum at level of alveolar bone margin Parallel lines are due to arrangement of the cemental collagen fibrils with their periodic striations in register Note relatively sparse cementum matrix Upper second premolar of 20 year old woman × 29 000

ce

G

20

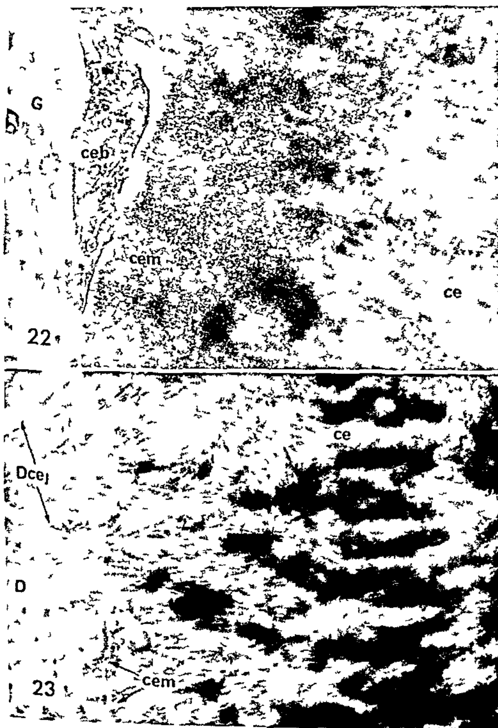
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## PLATE 12

### EXPLANATION OF FIGURES

22 Granular cementum matrix on the gingival surface of cementum The cementum matrix is closely related to a cytoplasmic process probably belonging to a cementoblast Upper second premolar of 20 year old woman × 40 000

23 Dentino cemental junction Note fraying of cemental fibrils near the junction with dentine Compare parallel arrangement of fibrils in cementum to haphazard arrangement of collagen fibrils in dentine Lower second deciduous molar of five year old boy × 14 000


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## › Development and Maintenance of Myelinated ›ue Cultures of Rat Trigeminal Ganglion[1]

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ABSTRACT Trigeminal ganglia of fetal or neonatal rats and mice have been cul tured on collagen-coated coverslips in Maximow assemblies The methods which proved optimal and the natural history of the resulting cultures are presented in detail Cultures formed abundant myelin remained healthy for at least three months and resembled previously described cultures of other primary sensory ganglia in most but not all respects New features included an exceptionally high requirement for glucose and apparently for other nutrients migration of some neuron somas and the occasional occurrence of stable but abnormally formed myelin segments The advantages and limitations of such cultures for experimental study of isolated nervous tissues are discussed

Tissue culture methods are now avail e which permit many fetal or neonatal ›ous tissues to undergo part of their mal differentiation *in vitro* and to be intained in this differentiated state for eks or months Such cultures are valu le experimental models for the study of iny properties of the intact nervous sys n This subject has been extensively re- wed by Murray (65) who has been a ider in the development of this experi ntal discipline

This paper reports our experience with e behavior in Maximow double-coverslip ltures of a part of the nervous system hich has not previously been cultivated *vitro* the trigeminal ganglion of late tal or neonatal rat or mouse Our tech ology was derived from that of Murray nd her school but a number of minor ew features appeared both in the specific iethods which proved optimal in our ands and in the natural history of our ultures A detailed description of our cul ure methods and results is therefore war anted as the indispensable baseline for he interpretation of experiments now in rogress utilizing these cultures to analyze roperties of peripheral nervous tissue (for xample Asbury Sidman and Wolf 66 and Winkler 65)

MATERIALS AND METHODS

Distilled water redistilled from glass was used for final rinsing of all glassware and to make up all culture media Glass ware other than coverslips was washed by Hanks metasilicate hydrochloric acid method (Paul 59) and rinsed in three changes of distilled and one of glass-dis tilled water Gold Seal brand coverslips were arranged in porcelain racks (Coors) boiled in metasilicate solution rinsed in running hot tap water immersed in pure nitric acid for half an hour then rinsed in running distilled and two changes of glass-distilled water All glassware was air dried and sterilized by dry heat

Tyrode s balanced salt solution (BSS) was made up and autoclaved without bi carbonate and sodium bicarbonate solu tion separately sterilized by filtration (Millipore Swinny adaptor) was added to complete the formula

Human placental blood (PS) was col lected from normal term deliveries After overnight clot retraction the serum was removed kept at room temperature for a week then tested for sterility and stored at 4 C Serum was expected to be clear

[1] S pported by grants NB 04600 nd NB 04792 from th N tion l In titute of Neurologi l Dise se and Blindne s.

and yellow specimens showing significant hemolysis (reddish or brownish hue) were rejected

Embryo extract (EE) was made by grinding nine day chick embryos in a ten Broeck grinder with half their volume of BSS incubating the mash at 36° for one hour and centrifuging it at 2 000 RPM for 30 minutes The supernatant was frozen in 0 5 ml aliquots which were thawed and recentrifuged on the day of use The final supernatant was expected to be opalescent After prolonged storage of EE the final supernatant became clear indicating deterioration Therefore fresh EE was made about every two months

The 22 mm round no 1 coverslips destined to carry cultures were coated on one side with a drop of dialyzed rat tail collagen solution (Bornstein 58) exposed briefly to ammonia vapor air dried for 30 minutes to one hour and stored in BSS

The first culture trials were made on trigeminal ganglia of mice several days old It proved more advantageous to use younger animals and to use the rat rather than the mouse The most reliable and abundant myelination in culture was obtained by using fetal rats of 18 to 19 days gestation However cultures of newborn mouse ganglia when successful were similar in all essential features

Non inbred Sprague Dawley rats were obtained from Charles River Laboratories or Gofmoor Farms

Rat fetuses were delivered aseptically by Caesarian section The calvarium was removed and the brain retracted caudally to expose the trigeminal ganglia on the floor of the middle cranial fossa The overlying dura was removed the nerves transected proximally and distally the ganglia removed and stripped of connective tissue Thorough stripping with disruption of the capsule seemed to favor prompt and abundant outgrowth good flattening of the explant and subsequent healthy development There was no advantage however in dividing a ganglion into two or more pieces Each suitably prepared ganglion was placed on a collagen coated coverslip bearing a drop (about 0 05 ml) of nutrient medium The culture was then incorporated into a Maximow double coverslip assembly and incubated at 35 5°C ± 0 5 in the lying drop position The drop was drained off and replaced twice weekly

Originally, the nutrient medium consisted of equal parts of PS EE ox serum ultrafiltrate (commercially obtained) and BSS mixed with sufficient 5% glucose to give a final concentration of 0 3% in the complete medium Before each feeding the cultures were washed by immersion in BSS After several modifications of the medium were tested it was found best to increase the final glucose concentration to 1 1–1 2% omit the ox serum ultrafiltrate and omit the washing The richer medium reduced the incidence of spontaneous degeneration of cultures which was thought to result from exhaustion of some nutrient in the lying drop The omission of washing reduced the incidence of bacterial contamination simplified and speeded the feeding procedure and had no effect on the development of the cultures

Maintenance of sterile technique has now been enormously simplified by carrying out all sterile operations in a hood through which flows a constant stream of filter sterilized air (White Rooms Inc.) This has eliminated the need for separate cubicles and gowns and masks

Living cultures were examined by bright field microscopy A long working distance oil immersion lens (Bausch and Lomb 40 × fluorite) was indispensable for high magnification microscopy through the double coverslip The development of cultures was unaffected by daily removal to room temperature for examination

Cultures to be fixed and stained were handled as whole mounts Some were fixed in 10% acrolein in M/10 phosphate buffer either for one hour at room temperature or overnight at 4 C and stained with sudan black B for myelin or with cresyl violet or toluidine blue for nuclei and Nissl substance Others were fixed in 10% formalin in BSS for a week or longer in the refrigerator and impregnated with silver by an adaptation of Holmes method for neurofibrils (Wolf 64)

For study of myelin development in *vivo* trigeminal ganglia of infant rats of various ages were fixed in 10% acrolein embedded in polyester wax (Sidman Mottla and Feder 61) sectioned at 8 μ and stained with Luxol Fast Blue

## RESULTS

_me thin neurites began growing out [of] the explants almost immediately [G]rowing neurites as long as 50 μ have [been] observed as early as six hours after [expl]antation (fig 1) At this stage of de[vel]opment the explants formed opaque [mas]ses approximately 1 mm in diameter [and] no neuron somas were observed out[side] the explant

[B]y one day after explantation the [radi]ally oriented outgrowth extended 0 5 [to 0]75 mm beyond the edge of the ex[plant] The outgrowing neurites were now [over]taken by a network of emigrating [Sch]wann cells and fibroblasts In addi[tion] a few neuron somas started to move [int]o the most proximal part of the out[gro]wth (fig 2) As the cultures matured [the]se neurons increased their distance [fro]m the explant Established cultures of[ten] contained a few healthy neuron somas [as] much as 300 to 400 μ beyond the origi[na]l boundary of the explant (fig 7)

During early development occasional [ma]crophages were observed ingesting [ery]throcytes and debris Macrophages be[ca]me far more numerous during the sub[se]quent days as the explant thinned out [an]d myelin formation commenced As the [ar]chitecture of the cultures stabilized [m]acrophages emigrated to the periphery or [fe]ll into the drop of medium as described [by] Bornstein and Murray (58) However [so]me macrophages remained throughout [th]e life of the cultures

By the second week *in vitro* the explant [be]came sufficiently thin that individual [n]euron somas could be distinguished with[in] its original boundaries Sometimes in[di]vidual somas could be distinguished in [a]ll regions of the explant but most somas [o]ccurred in clusters (fig 4) and the center [o]f the explant usually remained too thick [t]o resolve cellular detail (figs 7 9) Satel[li]te cells were always closely applied to [n]euron somas (figs 5 8) Neuron nuclei [w]ere clear and usually centrally placed [(]figs 4 5 8) and contained a prominent [n]ucleolus The living cytoplasm appeared [t]o contain evenly distributed small gran[u]les (figs 4 5) The Nissl bodies revealed [b]y fixation and staining (fig 8) were also [u]niformly distributed throughout the peri[k]aryal cytoplasm but were too large to correspond with the granules seen during life Neuron somas were somewhat oval The largest uninucleate neurons ranged from 30 to 35 μ in maximum diameter Neuron somas much smaller than this were usually not clearly seen during life Binucleate neurons were occasionally seen (fig 4) and were somewhat larger than uninucleate ones (largest diameter 50 to 60 μ)

The rat trigeminal ganglion *in situ* showed no myelin stainable by Luxol Fast Blue at birth Some segments of myelin were seen by this method in sections of ganglia of two-day old rats myelination was abundant at three and massive at four days of age According to Dixon (63) the majority of myelin sheaths in ganglia of four-day-old rats have mature ultrastructure

Cultures of trigeminal ganglia showed some myelin visible in the living state with the oil immersion objective as early as seven days *in vitro* Since these ganglia were explanted about three days before birth was expected this corresponds approximately to four days post natal age The earliest myelin (fig 3) appeared as single segments each with an associated Schwann cell nucleus Over the ensuing days additional segments appeared on scattered nerve fibers By 13 days *in vitro* over 90% of cultures contained some myelin segments and about 10% contained numerous axons possessing ten or more consecutive myelin segments During the ensuing two weeks 60 to 75% of cultures came to form large closely interwoven net works of myelinated axons some ran domly oriented some grouped in compact fascicles (figs 5 6 9 10) At the same time the diameter of individual axons and the thickness of their myelin sheaths both increased so that by four weeks *in vitro* myelin segments ranged from 0 5 μ to 5 μ in diameter Nodes of Ranvier could reg ularly and incisures of Schmitt Lanter mann could sometimes be identified in the living cultures but better photographs of these structures were obtained after fixa tion and staining (figs 11–14) Large myelinated axons were visible in living cultures at low magnification (10 × objec tive) and often branched at nodes of Ran vier (Fig 11) distal to the branch point

the fibers might or might not decrease in diameter

On one occasion an axon appeared traceable in a living culture from a neuron soma in the proximal outgrowth region to a myelin segment beginning 35 μ from that soma Almost invariably however the unmyelinated initial segments of axons were invisible during life so that given neuron somas could not be traced in this way to their myelinated axons Silver staining which revealed this initial segment was incompatible in our hands with the Luxol Fast Blue or sudan black B stains for myelin

After four weeks *in vitro* no new myelin segments appeared to be formed but the myelin continued to increase somewhat in thickness became more irregular in contour and developed multiple small invaginations and protrusions which were more numerous near nodes of Ranvier (figs 5 13) This wrinkling gradually increased with time and became very conspicuous in the oldest cultures Most cultures were terminated between 30 and 60 days *in vitro* but others set aside to test their longevity regularly attained three months and occasionally four months of age *in vitro*

Silver staining revealed some additional structural features (fig 15) The total number of axons seen in any given culture after silver staining was much larger than the number of myelinated axons seen in the same culture during its life showing that the majority of axons were unmyelinated In addition to the large neuron somas located primarily at the periphery of the explant where they had been recognized during life numerous small neuron somas (10 μ or less in greatest diameter) were found in the center of the explant where they were only recognized after staining Correlations between soma diameter and axon diameter could not be investigated because the extensive branching and interweaving of axons frustrated our attempts to trace them through the thick central regions of cultures

The initial segments of axons were straight or had at most one or two turns or angulations Sometimes the initial segments were broadly tapering and contained an extension of the delicate lightly stained neurofibrillar network of the soma rather than being solid black like typical mature axons Blunt, lobulated protrusions from the soma were sometimes seen but the tortuously folded intracapsular networks" and "glomeruli of the initial segments described by Ramon y Cajal (3 and others were not recognized in silver stained cultures However the initial segments of axons could not be seen at all in many instances and it is possible the glomeruli and intracapsular networks existed in our cultures but were refractory to staining by Holmes method or were obscured by the high optical density of successful Holmes preparations The majority of the neurons were unipolar but many bipolar and some multipolar ones also were seen

We observed in some cultures an abnormality of myelin formation (fig 14 consisting of grotesque asymmetrically ballooned myelin segments along the course of an axon which elsewhere might bear normally formed myelin Often many axons in a particular fascicle were affected The ballooned segments appeared to be abnormal in shape when first discovered and to survive intact for as long as the normally formed segments This abnormality was most frequent in those cultures showing the most precocious and abundant myelin Silver preparations of these cultures showed no abnormality of the axons

Sporadic instances of spontaneous degeneration were observed One type of degeneration primarily involved myelin which over a period of several days would break up into refractile granules and be phagocytosed The neurons Schwann cells and fibroblasts retained normal morphology If such cultures were maintained for another 2 to 3 weeks in this unmyelinated state a few segments of myelin might appear presumably regenerated in culture Another type of degeneration consisted of overnight necrosis of all cellular elements in the culture with myelin retaining its structure for an additional 1 2 days before breaking up Both of the types of degeneration were reduced in frequency though not eliminated by the modifications of the medium described under METHODS

our days was the longest permissible rval between feedings Cultures left d for five days or longer began to show ird type of degeneration Myelin seg its developed smooth symmetrical oval tations broke up into ovoids and were gocytosed neuron somas swelled nu moved peripherally and the neurons Feeding before this process was plete would preserve those neuron ias and myelinated axons which were intact but the myelinated axons ch had developed oval dilatations would ceed to complete degeneration despite ewal of the nutrient medium

Another very troublesome type of de eration resulted from development of all holes or tears in the collagen film or alized detachment of the film from the derlying coverslip Once the collagen n was broken the tension exerted on it the maturing connective tissue cells in culture led to rapid increase in size of break culminating in physical disrup- n of the entire culture These collagen aks could be initiated by any contact an instrument or glass surface with the llagen but also occurred seemingly ontaneously They were reduced in fre ency by the use of highly viscous well alyzed collagen but it was necessary to oid dialyzing the collagen to the point here it began to gel within the dialysis ig Fragments of gelled collagen when corporated into a collagen film on a verslip served as foci for the initiation subsequent collagen breaks Careful dis ction of connective tissue from the gan ia at the time of explantation and the se of younger animals also reduced the requency of collagen breaks Breaks were much more troublesome with mouse cul ires perhaps because the small size of mouse ganglia increased the difficulty of moving connective tissues

## DISCUSSION

Cultures of rodent trigeminal ganglia re emble cultures of other sensory ganglia in nost but not in all respects A require nent for high glucose concentration in the medium and a preference for collagen gel is solid substrate are shared by trigeminal and spinal ganglia and central nervous issue of rats mice cats and humans but not by avian or amphibian nervous tissues (Bornstein and Murray 58 Murray 65 Peterson and Murray 55 Peterson Crain and Murray 65)

These characteristics seem therefore to be a function of phylogenetic rather than histologic class The unusually high con centration of glucose and apparently of other nutrients required by our cultures of trigeminal ganglia may simply reflect the size of the cultures It is our impres sion that the sheer bulk of neuron cyto- plasm present on a single coverslip and therefore fed by a single drop of me dium is much greater in healthy cultures of trigeminal ganglia (see figs 9 10) than in similar and equally healthy cultures of cerebellum pons spinal ganglion spinal cord or cerebral cortex (Wolf 64 and unpublished observations) It is unneces sary therefore to postulate any metabolic peculiarity of the trigeminal ganglion to explain the nutritional requirements shown by our cultures although the pos sibility remains that such peculiarities may some day be detected by other means

The movement of neuron somas in our cultures away from the site of explanta tion could be interpreted as mere passive spread of the explant but it seems more likely that it represents active migration Murray and Stout ( 47) observed active migration of postganglionic adult sympa thetic neuron somas *in vitro* those cells were embedded in clotted plasma and pre sumably had to overcome more passive resistance than cells growing on the sur face of collagen gel More recently Peter son Crain and Murray ( 65) observed that when fragments of fetal spinal cord were explanted on collagen film together with their attached dorsal root ganglia the gan glia migrated away from the cord frag ments spinning out a tract of nerve fibers corresponding to the proximal dorsal root *in vitro* Entire ganglia migrated over 0 5 mm during three months *in vitro* Our cultures of trigeminal ganglia were set up without spinal cord or brainstem trigemi nal neurons like those of sympathetic gan glia are apparently able to migrate with out extrinsic influences

The one major morphological feature of the trigeminal neuron in situ which ap pears to be lacking in cultured ganglia is

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the coiling of the initial segment of the axon into an elaborate intracapsular net work or glomerulus Other characteristics of the trigeminal ganglion such as the sizes and shapes of neuron somas their relationships to satellite cells the relation ship of myelin segments to Schwann cells the form and distribution of nodes of Ranvier and incisures of Schmitt Lantermann are closely similar *in situ* and *in vitro* The maximum diameter of myelin segments in rat trigeminal cultures is the same as found in the rat trigeminal ganglion *in situ* (Dixon 63) and the wrinkles and irregularities appearing in cultured myelin sheaths with advancing age *in vitro* cor respond to those observed by Webster and Spiro ( 60) in sciatic nerve of normal adult rats The cultured neurons are of course abnormal in the sense of being isolated from their peripheral end organs from their postsynaptic target cells in the CNS and from much of their normal mesodermal environment However the isolation itself confers on these neurons the advantages of accessibility to direct microscopic observation and chemical or mechanical manipulation Isolation also simplifies the interpretation of experimen tal findings observed reactions must be intrinsic to those cells present in the cul ture rather than being possibly influenced by the participation of distant organs as may be the case *in situ*

The usefulness of such cultures as test objects to study disease mechanisms has been well established (Bornstein 63 Winkler 65) and requires no extensive discussion here A note of caution is in troduced by the presence of some abnor mally formed myelin segments and the occasional occurrence of spontaneous de generation in even our best cultures Rig orous controls are essential to establish that a deleterious effect obtained in an ex periment concerned with disease mecha nisms has actually been produced by that aspect of the experimental challenge which is relevant to the mechanisms of the disease under study

Coverslip cultures of nervous tissues show some instructive limitations in other types of experiments For example as part of a recent study of porphyrin pro duction by nervous tissues *in vitro* (As bury Sidman and Wolf 66) meas ments were desired of porphyrin prod and of the activity of certain enzymes in volved in porphyrin synthesis One and sometimes two, 10 µl aliquots of the drop of medium could be recovered analysis although complete recovery impossible because of adherence of me dium to the small and large coverslips The total enzyme activity of individ cultures appeared to be increased ab 100% by appropriate chemical stim tion However no significance could attached to this increase because the amount of actively metabolizing tissue per culture was variable and uncertain Th wet or dry weight and the protein con of different cultures varied as much a 100% and even these measurements w rendered meaningless by the inevita inclusion of collagen film and dead or in cells in uncertain proportions along w living nervous tissue DNA measur ments would also include dead and in cells and would fail to reflect differen in cytoplasmic volume between the la neurons and the much smaller sa el and Schwann cells Until means are fou to solve or circumvent this measurem problem cultures of nervous tissue will refractory to quantitative enzymolog studies

In the same study of porphyrin metab lism fluorescence microscopy of cultur was undertaken in an attempt to de mine the precise cell or structure in whi the porphyrin was localized Porph fluorescence could be seen at high m nification but details of cell structure we almost completely obscured by backgro fluorescence from other cells lying ab and below the plane of focus Heal neuron somas tended to occur in clust three and four deep (see fig 4) Even t somas and myelin segments that appear by conventional microscopy to be well i lated in the outgrowth zone proved to completely enveloped by layers of Schwa and connective tissue cells whose diffu fluorescence obscured detail This env opment might also be expected to incr the difficulty of other experimental man ulations such as the insertion of mic electrodes into neurons under direct m croscopic visualization Indeed both Cra

and Hild and Tasaki (62) have re ed upon the great difficulty of ac lishing microelectrode penetrations iltured neurons whether grown in ia clot or on collagen gel These trou me multiple layers of cells cannot be ed as merely a nuisance which might liminated by some improvement in re methodology They are part and el of the conditions for optimum dif itiation and survival of neurons *in*

Neurons seem to require intimate eathment and tridimensional support heir companion cells for healthy sur Methods developed thus far for the tion of cultured neurons all have en d some compromise with respect to rentiation and stability (for example al 56) The best isolated neurons in ptionally thin cultures are *in this* e not as reliable models of the be or of the neuron *in situ* as their less ssible fellows in thicker cultures en more precise information becomes lable about specific macromolecules ured by various cell types for survival differentiation — such as the sympa ic nerve growth factor (Levi Montal 64) which must represent the first covered example of a large class of spe trophic factors — it may become pos e to maintain totally isolated differenti d neurons *in vitro* At present healthy intenance of neurons *in vitro* requires healthy maintenance of their sur nding matrix of associated cells The blems thus presented are in the same is as the problems which the nervous tem *in situ* has always posed for the perimenter because of its extraordinary erogeneity Limitations of present cul e techniques serve to demonstrate the portance of this heterogeneity at the el of the intimate organization of ner us tissue

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the coiling of the initial segment of the axon into an elaborate intracapsular net work or glomerulus Other characteristics of the trigeminal ganglion such as the sizes and shapes of neuron somas their relationships to satellite cells the relation ship of myelin segments to Schwann cells, the form and distribution of nodes of Ranvier and incisures of Schmitt Lantermann are closely similar *in situ* and *in vitro* The maximum diameter of myelin segments in rat trigeminal cultures is the same as found in the rat trigeminal ganglion *in situ* (Dixon 63) and the wrinkles and irregularities appearing in cultured myelin sheaths with advancing age *in vitro* correspond to those observed by Webster and Spiro (60) in sciatic nerve of normal adult rats The cultured neurons are of course abnormal in the sense of being isolated from their peripheral end organs from their postsynaptic target cells in the CNS and from much of their normal mesodermal environment However the isolation itself confers on these neurons the advantages of accessibility to direct microscopic observation and chemical or mechanical manipulation Isolation also simplifies the interpretation of experimental findings observed reactions must be intrinsic to those cells present in the culture rather than being possibly influenced by the participation of distant organs as may be the case *in situ*

The usefulness of such cultures as test objects to study disease mechanisms has been well established (Bornstein 63 Winkler 65) and requires no extensive discussion here A note of caution is introduced by the presence of some abnormally formed myelin segments and the occasional occurrence of spontaneous degeneration in even our best cultures Rigorous controls are essential to establish that a deleterious effect obtained in an experiment concerned with disease mechanisms has actually been produced by that aspect of the experimental challenge which is relevant to the mechanisms of the disease under study

Coverslip cultures of nervous tissues show some instructive limitations in other types of experiments For example as part of a recent study of porphyrin production by nervous tissues *in vitro* (Asbury Sidman and Wolf 66) ments were desired ... and of the activity of certain enzymes volved in porphyrin synthesis One ... sometimes two 10 µl aliquots of the h... drop of medium could be recovered ... analysis although complete recovery ... impossible because of adherence of ... dium to the small and large ... The total enzyme activity of indiv... cultures appeared to be increased 100% by appropriate chemical stim... tion However no significance coul... attached to this increase because amount of actively metabolizing tissu... culture was variable and uncertain wet or dry weight and the protein of different cultures varied as muc... 100% and even these measurements rendered meaningless by the inev... inclusion of collagen film and dead or ... cells in uncertain proportions along ... living nervous tissue DNA m... ments would also include dead and ... cells and would fail to reflect dif... in cytoplasmic volume between the la... neurons and the much smaller satellit... and Schwann cells Until means are fo... to solve or circumvent this measureme... problem cultures of nervous tissue will ... refractory to quantitative enzymolog... studies

In the same study of porphyrin metab... lism fluorescence microscopy of cultu... was undertaken in an attempt to ... mine the precise cell or structure in whic... the porphyrin was localized Porphyr... fluorescence could be seen at high mag... nification but details of cell structure we... almost completely obscured by background fluorescence from other cells lying ... and below the plane of focus Heal... neuron somas tended to occur in clu... three and four deep (see fig 4) Even the somas and myelin segments that appeared by conventional microscopy to be well iso... lated in the outgrowth zone proved to b... completely enveloped by layers of Schwan... and connective tissue cells whose diffu... fluorescence obscured detail This env... opment might also be expected to ... the difficulty of other experimental manip... ulations such as the insertion of micr... electrodes into neurons under direct m... croscopic visualization Indeed both Cra...

;rand Hild and Tasaki (62) have re -d upon the great difficulty of ac -ishing microelectrode penetrations -ltured neurons whether grown in ia clot or on collagen gel These trou ne multiple layers of cells cannot be ; d as merely a nuisance which might ,minated by some improvement in re methodology They are part and l of the conditions for optimum dif tiation and survival of neurons *in*

Neurons seem to require intimate eathment and tridimensional support heir companion cells for healthy sur Methods developed thus far for the tion of cultured neurons all have en d some compromise with respect to rentiation and stability (for example al 56) The best isolated neurons in ptionally thin cultures are in this e not as reliable models of the be ior of the neuron *in situ* as their less essible fellows in thicker cultures en more precise information becomes ilable about specific macromolecules uired by various cell types for survival l differentiation — such as the sympa tic nerve growth factor (Levi Montal i 64) which must represent the first covered example of a large class of spe c trophic factors — it may become pos le to maintain totally isolated differentı d neurons *in vitro* At present healthy intenance of neurons *in vitro* requires : healthy maintenance of their sur inding matrix of associated cells The oblems thus presented are in the same iss as the problems which the nervous stem in situ has always posed for the periménter because of its extraordinary terogeneity Limitations of present cul re techniques serve to demonstrate the iportance of this heterogeneity at the el of the intimate organization of ner ous tissue

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## PLATE 1

### EXPLANATION OF FIGURES

Living cultures of rat trigeminal ganglion showing features of early development

1 Six hours *in vitro* A few delicate neurites are already growing out of the explant × 485

2 Two days *in vitro* Some neuron somas have started to move away from the edge of the explant × 725

3 Seven days *in vitro* A myelin sheath has appeared around an axon Both the myelin and the underlying axon appear smaller in diameter than the mature ones shown at the same magnification in Plate 2 × 725


1


2


3

## PLATE 1

### EXPLANATION OF FIGURES

Living cultures of rat trigeminal ganglion showing features of early development

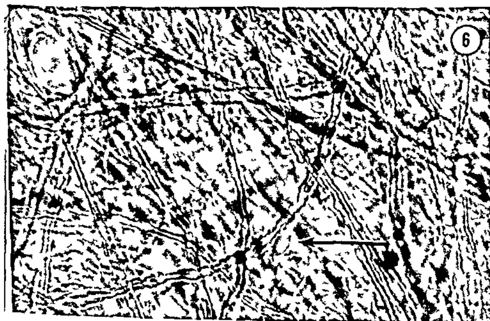
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4


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## PLATE 2

### EXPLANATION OF FIGURES

Living neuron somas and myelin sheaths in mature cultures

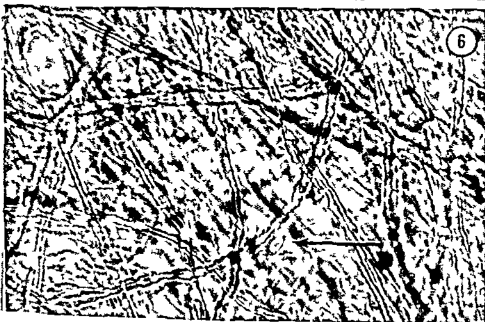
4 Twenty five days *in vitro* A cluster of neuron somas Note the centrally placed nuclei and uniform small cytoplasmic granules One binucleate neuron is present (arrow) Satellite cell nuclei are seen between neuron somas Myelinated axons course through the lower part of the field × 725

5 Isolated neuron soma in the proximal outgrowth zone The apparent bumps on its surface are the nuclei of satellite cells surrounding it Segments of myelin course in and out of the plane of focus × 725

6 Field of myelinated axons The Schwann cell associated with one of the myelin segments is in focus (arrow) This myelin segment and some others in the picture show conspicuous wrinkling × 725


4


5


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7
8

## PLATE 3

### EXPLANATION OF FIGURES

Trigeminal ganglion 29 days *in vitro* Stained with toluidine blue

7 Survey view The edge of the explant above proximal outgrowth below Several neuron somas (arrows) occupy positions 200 to 400 μ away from the explant × 200

8 High magnification view of the neuron in the center of figure 7 showing centrally placed nucleus uniformly distributed clumps of Nissl substance and on the upper right margin a closely apposed satellite cell nucleus × 1 350

9



## PLATE 4

### EXPLANATION OF FIGURES

Trigeminal ganglion 30 days *in vitro* Stained with sudan black B

9 Survey view of entire culture The explant is surrounded by a dense halo of outgrowing myelinated axons some coursing at random some especially in the upper right quadrant travelling in compact parallel fascicles Macrophages intensely stained because of their phagocytosed fat droplets are scattered in the outgrowth About × 23

10 Higher magnification of the area bracketed in figure 9 A lightly myelinated area was chosen to permit individual myelinated axons to be distinguished These two figures show how much healthy nervous tissue may be present on a single coverslip in the best cultures of trigeminal ganglion About × 92


11
12


13


14

## PLATE 5

### EXPLANATION OF FIGURES

Features of myelin sheath structure at high magnification Cultures stained with sudan black B

11 Bifurcation of a myelinated axon at a node of Ranvier The segment proximal to the bifurcation shows two incisures of Schmitt Lantermann (arrows) Three additional myelinated axons are in the field Forty two days *in vitro* × 725

12 Another incisure of Schmitt Lantermann Thirty days *in vitro* × 1 000

13 Extensive wrinkling of myelin adjacent to a node of Ranvier Thirty days *in vitro* × 1 000

14 Abnormal grotesquely ballooned myelin segments Note that the adjacent myelin segment on the same axon (arrow) and other segments in this field are normally formed Thirty days *in vitro* × 400


15
P
N
A

## PLATE 6

### EXPLANATION OF FIGURE

Structural features revealed by silver impregnation

15 Trigeminal ganglion 45 days *in vitro* Holmes silver method Note the extremely numerous axons more numerous than myelin sheaths in any corresponding field examined during life or after sudan black staining The large neuron in the center has a protoplasmic extension of its soma (P) Its axon appears as a solid black line following the left margin of the cell soma and then turning away to the left of the field Another large neuron near the bottom of the field has an axon which contains a loose neurofibrillar network and makes a hairpin turn close to the soma (A) No greater tortuosity of the initial segment was observed in silver impregnated cultures Numerous small neurons under 20 $\mu$ in diameter are also present in this field (N) Small neurons such as these were not usually identified during the life of the culture Approximately $\times$ 1 000


15
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15
P
N
A

# ects of Triton WR 1339 on the Rat Yolk Sac Placenta [1]

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ABSTRACT The cytochemistry and ultrastructure of the lysosomal area of the rat s yolk sac placenta was studied at various stages of development The lysosomal area located at the apical end of the yolk sac epithelial cell between the microvillous border and the nucleus consisted of dense bodies that persisted from day 10 of pregnancy through gestation and multivesicular bodies that are present only through day 12 Acid phosphatase activity was restricted to the dense material and to the membranes of this area Two types of pinocytotic vesicles were present in all stages of development One was a tubular structure containing dense material and the other a saccular clear structure containing a "fuzz lining and membrane remnants Possible relationships between the pinocytotic vesicles and the dense and multi vesicular bodies were considered

The injection of Triton WR 1339 on day 11 of pregnancy resulted in a vacuolation of the dense and multivesicular bodies This vacuolation began 15 minutes after injection and maximum effect was reached within 12–24 hours A reorganization of the lysosome area occurred on days 15–16 in those animals in which pregnancy continued to term This reorganization consisted of an increase in the dense material within the vacuoles and a reduction in vacuole size The vacuoles remained distended in those animals in which death and resorption of the fetus occurred The first cellular changes associated with death occurred between 48–60 hours post injection The microvilli became shorter thicker and reduced in number the pinocytotic vesicles disappeared from the cell surface the endoplasmic reticulum became dilated and vesiculated and vesicles were formed from the cristae of the mitochondria

With the injection regimen used in this study 25% of the litters were resorbed while the remainder of the pregnant rats delivered viable fetuses at term

Termination of pregnancy and resorp[tio]n of the rat fetus and placenta can be [in]duced in many ways but the mechanism [of] this phenomenon has not been studied [It] may be that the lysosomes of the fetus [an]d placenta play a role in the digestion of [th]e conceptus since lysosomes have been [im]plicated in other types of tissue degen[er]ation (de Duve 63 Weissmann 65) [A] previous histochemical study of the dis[tr]ibution of lysosomes in ethionine induced [re]sorption of conceptuses gave evidence in [su]pport of this idea in that the size and [d]istribution of these organelles appeared [t]o change with resorption (Schultz and [S]chultz 62)

The structure in the rat conceptus with [t]he most pronounced acid phosphatase ac[t]ivity the reference enzyme for lysosomes [w]as the yolk sac placenta Acid phospha[t]ase activity was found to be localized in [t]he supranuclear region of the visceral [y]olk sac epithelial cell (Wislocki Deane [a]nd Dempsey 46 Padykula 48 Schultz [a]nd Schultz 62) This region has also [b]een shown to contain a heavy concentration of diastase resistant carbohydrate (Wislocki and Padykula 61) which has been related to lysosomes (Koenig 62) Other studies have shown that the apical region of the yolk sac cell is a region in which foreign materials especially vital dyes are concentrated (Everett 35 Wislocki Deane and Dempsey 46 Wilson Beaudoin and Free 59 Al Abbass and Schultz 66) For this reason it has been suggested that the yolk sac cell acts as a placental barrier to prevent foreign materials from reaching the fetus (Wislocki Deane and Dempsey 46 Al Abbass and Schultz 66)

Fine structural studies of the supranuclear region of the visceral yolk sac epithelial cell have shown the region to contain

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[3] Pr sent ddres Dep rtment of An t my Un er sity of T nn see Medic l Units, Memphis

[4] S pported by R arch Career Dev l pment Awa d K3 HD 7680

post fixed for 45 minutes in cold buf 1% osmium tetroxide pH 7 4 The s were dehydrated in methanol in ed with a propylene oxide Epon mix and embedded in Epon 812

control morphology a number of es were fixed in buffered glutaralde (3–6 25%) pH 7 4 post fixed in ed 1% osmium tetroxide pH 7 4 mbedded as described above As a for the specificity of localization of on product a number of samples fixed in varying concentrations of aldehyde (1–6 25%) pH 7 35 and d for acid phosphatase activity after ethod of Miller (62) but because of capriciousness of this method in yolk ells it was not used routinely

in sections 600–900 Å were cut with or diamond knives on the LKB or r Blum MT2 ultramicrotome stained 7 5% uranyl acetate and 0 2% lead te and examined with the Phillips electron microscope at 60 KV Photo- hs were taken on Kodak Contrast s

RESULTS

*Control observations*

he normal fine structure of the lyso- al area of the yolk sac epithelial cell studied at various stages of develop- nt and was considered in relation to the ribution of acid phosphatase activity in this region Particular attention given to the cellular elements pino- tic vesicles dense bodies and multi cular bodies

*Pinocytotic vesicles* There were two s of pinocytotic vesicles present in all es of development studied One type tubular containing very dense mate These vesicles retained their tubular earance after they were separated from cell surface sometimes forming U aped structures and ultimately ap- ared to fuse with dense bodies located the apical portion of the yolk sac cell g 3 12) Their contents were of the he opacity as the material present in adjacent dense bodies These struc es have been referred to as canaliculi Vislocki and Padykula 61)

Pinocytotic vesicles of the second type ere larger than the tubular type and were expanded distally so that they appeared to be saccular Each vesicle contained an outer ring of slightly dense material adja cent to the membrane ("fuzz lined" Car penter and Ferm 66) and membrane remnants were frequently found in the lumen (fig 2 3 8 12) The type of mem brane remnant in the clear pinocytotic vesicle was also found free in the lumen of the yolk sac (fig 1 8 10 16) These vesicles were found at various depths within the supranuclear region of the cell

Neither of these vesicles reacted for acid phosphatase except in cases where reaction product was also present on the microvilli and upon the nuclear membrane This reaction has been said to be an arti fact (Novikoff 63)

*Dense bodies* In all of the stages stud ied the yolk sac epithelial cells contained large dense bodies varying in size (2–6 μ in diameter) and opacity (fig 1 2 3 6 7) Those bodies nearest the cell surface were much more opaque than those more proximally located A gradual reduction in the size of the dense bodies occurred in later stages of pregnancy By day 20 the dense bodies ranged in size from 1–3 μ and occupied the greater portion of the apical part of the cell The membranes of mito- chondria and the endoplasmic reticulum fused with the smooth membrane of the dense bodies Frequently membrane rem nants were found in the space between the dense material and the limiting membrane of the dense bodies (figs 2 3 6) Projec tions of cytoplasm containing free ribo- somes often extended into the area

Reaction for acid phosphatase was ob- served in the dense bodies located the greatest distance from the cell surface and in the cytoplasm between these bodies (figs 1 2) Reaction product was often lacking or sparsely distributed in the dis tally located dense bodies

*"Multivesicular" bodies* In the younger stages days 10 and 11 the epithelial cells of the yolk sac had relatively clear vacu oles containing disorganized membrane remnants myelin figures and scattered particles of dense material On day 12 these vacuoles 3–9 μ in diameter were filled with large numbers of small spher ical vesicles interspersed within the dense material (figs 3 4 5) Frequently the

dense and fuzz lined vesicles the membranes of which are continuous with the plasma membrane and several types of vacuoles containing dense material and vacuolated clear areas (Wislocki and Dempsey '55 Wislocki and Padykula 61) Materials injected into the uterine cavity were absorbed by the vesicles of the yolk sac cell an became concentrated in the vacuoles in the supranuclear region of the cell (Luse 57 Carpenter and Ferm 66) The vacuoles then correspond to the digestive vacuoles of the lysosome concept (de Duve 63) If acid phosphatase activity were to be demonstrated within the vacuoles this would substantiate the idea that they are lysosomal in character

To test the hypothesis that lysosomes may be directly involved in necrosis of the conceptus (Schultz and Schultz 62), Triton WR 1339 a non ionic detergent known to labilize the lysosomes of other tissues *in vivo* (Wattiaux, Wibo and Baudhuin 63) was injected into pregnant rats A histochemical study had demonstrated that 20–60% of the rat litters were resorbed after Triton injection and that the most marked morphological change in the conceptus was a pronounced vacuolation of the apical region of the epithelial cells of the visceral yolk sac These vacuoles were ringed by acid phosphatase activity and by non glycogen PAS reacting material suggesting lysosomal origin (Schultz and Schultz 66)

This report presents cytochemical and fine structural observations of the lysosomal area of developing normal yolk sac cells the vacuolation of this area following Triton injection and the ensuing changes associated with survival or death of the conceptus

## MATERIALS AND METHODS

*Injection regimen* Sprague Dawley rats were injected intravenously (vena cava inferior) on day 11 of pregnancy with 200 mg Triton WR 1339 in 1 ml rat Ringer's solution 300 mg Triton in 1 5 ml Ringer s or 400 mg Triton in 2 ml Ringer s solution Control animals were injected with an equivalent amount of rat Ringer s solution Conceptuses were obtained by laparotomy on days 11, 12 13 14 15 16 and 20 of pregnancy A total of sixty yolk sacs from 29 control rats and 87 yolk sacs fr- 50 experimental animals was used in t- study

Another series of experiments in- intravenous injections of 200 mg Tri- WR 1339 on day 12 of pregnancy and re- moval of conceptuses by laparotomy a- 30 60 120 240 and 360 minute inter- after injection A minimum of six y- sac samples were taken at each time in- terval

At the time of injection the inver- of the yolk sac had been completed, - chorio allantoic placenta had been est- lished, and the parietal yolk sa- - Reichert s membrane were intact

*Laparotomy* Consecutive samples w- taken from individual animals at the f- time intervals i e at a specific time - conceptuses were removed by laparo- the pregnancy allowed to continue a- each of the next time intervals the pro- dure was repeated In this manner - critical time after Triton treatment - followed by conceptus death or surv- could be determined with fair accur- Approximately 75% of the Triton tr- animals handled this way delivered l- fetuses at term Laparotomy did not - duce resorption in the control anim-

*Sample preparation* The folded por- of the visceral yolk sac placenta (thro- day 15 of pregnancy) was dissected f- the conceptus in cold 0 25 M sucrose - cut into small pieces approximately 5 - × 5 mm × 15–50 µ The samples w- fixed for 12 hours in either cold F- (Holt and Hicks 61) or Pease s (- fixative stored overnight in cold buffer- sucrose solution pH 7 3 rinsed with - 7 5% sucrose and reacted for acid - phatase activity with Daem s modifica- of Gomori substrate pH 5 (Daem - for 20 minutes at 37 C Thicker m- branes from older conceptuses (16- day) were fixed whole chopped in - 60 µ slices with the McIlwain mechanical tissue chopper (McIlwain and Buddle - Smith and Farquhar 63) and reacted f- acid phosphatase activity Duplicate sa- ples were inhibited by incubation in G- mori substrate containing 0 01 M NaF

After reaction the tissues were washed in 0 05 M acetate buffer containing 4- formaldehyde and 7 5% sucrose pH -

eared from the apical border of the the endoplasmic reticulum became .ted with an increased accumulation of material within the cisternae and ill vesicles were formed from the cris of the mitochondria (figs 16 17 18 The vacuolated bodies remained ger than those of yolk sac cells which vived the Triton effect The acid phos itase reaction was localized upon the ques of dense material and on the mem ne of the vacuoles (figs 16 17)

At 60–72 hours after injection the early dences of approaching cell death were anced and were accompanied by pyk of the nucleus and a total disorgani ion of the membranes in the apical ion of the cell The apical region of the became filled with many small vesi some of which had ribosomes asso ted with them and were probably de d from the endoplasmic reticulum gs 18 19) The vacuolated dense and ultivesicular" bodies became very large nd occupied the major portion of the pical region of the cell

When a cell could no longer be recog ized morphologically as a yolk sac epithe al cell the acid phosphatase activity was ill present and there was no change in he distribution of the reaction product en in cases of most advanced disorgani ation In the final stages of degeneration ie cells ruptured and the cell contents ere expelled into the extracellular area

DISCUSSION

The histology histochemistry fine struc ure and absorptive function of the yolk ac epithelial cell have been described in etail (Wislocki Deane and Dempsey 46 Amoroso 58 Wislocki and Padykula 61 Al Abbass and Schultz 66) These studies demonstrated that the apical region of the visceral yolk sac cell was absorptive in function had localized acid phosphatase activity and contained proteinaceous drop ets and non glycogen PAS reacting sub tances These structural and biochemical characteristics of the lysosome have been stablished during the last few years This study has verified the lysosomal and ab sorptive nature of the supranuclear area of the yolk sac cell and we have attempted to relate this area with the survival or death of the cell under adverse conditions A complete review of current concepts of the lysosome function has been presented by Weissmann (65)

The presence of acid phosphatase in the transitory "multivesicular" bodies and the proximal dense bodies indicated that they were lysosomes (Novikoff 63) The suc cessive transformation of the autophagic vacuoles of days 10 and 11 to multivesic ular bodies on days 12 and 13 and the transformation of multivesicular bodies to dense bodies in later stages of pregnancy corresponded to similar transformations in the lysosomal areas of other tissues (Gor don Miller and Bensch 65) Electrophor etic studies on the yolk sac have shown changes in acid phosphatase distribution between days 10 and 11 and between days 13 and 14 of pregnancy (Johnson and Spinuzzi 66) These days coincide with the time of occurrence of morphological changes in the lysosomes of the yolk sac epithelial cell It may be that the acid phosphatase in the lysosomes changes mo lecularly in conjunction with changes in fine structure

We have assumed that the vacuolation of all of the bodies in the supranuclear region of the cell was due to the absorption and accumulation of the Triton WR 1339 since the detergent administration was the only imposed variable in the experimental conditions This area has been shown to accumulate colloidal gold (Luse 57) and thorotrast (Carpenter and Ferm 66) It therefore seemed reasonable to assume that it was also capable of incorporating Triton Vacuolation of the lysosomes of the liver following Triton WR 1339 ad ministration has been demonstrated (Wat tiaux Wibo and Baudhuin 63) and the Triton filled vacuoles of the liver were mor phologically similar to those of the yolk sac cell

The marked increase in resorption fol lowing the administration of Triton was evidence that the detergent was detrimen tal to the conceptus Although it was clear from this study that Triton had a marked effect upon the morphology of the yolk sac placenta of resorbing animals we do not know whether the death of the yolk sac was a cause of or a result of the inter ruption of pregnancy Ultrastructural stud

membranes of mitochondria and endoplasmic reticulum fused with the surrounding membrane of the vacuoles (fig 4) The lack of organization of the membrane remnants within the vacuoles at days 10 and 11 was characteristic of autophagic vacuoles of other tissues while the uniformity in size and appearance of the inclusions of the vacuole on day 12 was similar to that of the multivesicular bodies of other tissues (Novikoff, 63 Novikoff Essner and Quintana, 64 Gordon, Miller and Bensch 65) We have referred to both structures as multivesicular bodies The multivesicular bodies disappeared completely by the fourteenth day and therefore were interpreted to be transitory structures in the differentiation of the lysosomal area of the yolk sac epithelium

The acid phosphatase activity was associated with the dense material with the vesicles and with the outer limiting membrane of the multivesicular body Acid phosphatase activity also appeared in the cytoplasm between these inclusions and the dense bodies

*Disposition of other organelles* Great numbers of mitochondria were found clustered about the lysosomal area (figs 2 3 4) The endoplasmic reticulum became more regularly disposed in the later stages of development and frequently contained dense material similar in opacity to that of the dense bodies (fig 15) The Golgi apparatus was localized in the perinuclear region and did not appear to be related to the lysosomal area None of these structures reacted for acid phosphatase

### *Observations following injection of triton*

In 75% of the pregnant rats injected with Triton WR 1339 the conceptuses survived any detrimental Triton effects and live fetuses were delivered at term In 25% of the pregnancies the conceptuses died and were resorbed

*Survival* The time before response of the yolk sac cell to Triton injection varied from animal to animal Some yolk sacs showed increased vacuolation within the dense and multivesicular bodies 15 minutes after injection while others did not show this until two hours after injection The maximum 'swelling" and vacuolation effect was obtained 4–6 hours after injection (fig 9)

Within 12 hours after injection the membrane remnants of the multivesicular bodies (9–17 μ) had disappeared almost entirely The opaque material of both the multivesicular bodies and the dense bodies appeared to be pushed to the periphery of the vacuoles where it formed dense plaques (figs 10 11 12) Fusion of the membranes of mitochondria and the endoplasmic reticulum with the membrane of the vacuoles was common (fig 12)

Acid phosphatase reaction [illegible] confined to the dense plaques the membrane of the vacuoles and the cytoplasm between vacuoles (fig 10 11)

The large clear vacuoles [illegible] through days 13 and 14 of pregnancy [illegible] membrane remnants and myelin [illegible] began to reappear in the lumen of the vacuoles Random dead cells and [illegible] from lysed cells were found on these [illegible] although the fetus was alive at the [illegible] the samples were taken By the fifteenth and sixteenth days, the vacuoles [illegible] come smaller and again began to contain large amounts of dense material (figs [illegible] 14) By the twentieth day the yolk sac [illegible] appeared similar to those of the controls with the exception of small clear [illegible] lated areas within the dense bodies [illegible] the presence of a larger amount of residual membrane (fig 15)

Occasionally after Triton treatment the intercellular spaces between epithelial [illegible] became dilated and disorganized and [illegible] elin figures and membrane remnants [illegible] found in these spaces (fig 16) At [illegible] time was there a measurable change [illegible] the size distribution or appearance of the dense or clear pinocytotic vesicles [illegible] brane remnants within the clear [illegible] totic vesicles did not disappear upon Triton injection as did those of the multivesicular body

*Death* All Triton treated yolk sac cells whether destined to survive or to [illegible] were morphologically similar through the 48 hour interval after injection The [illegible] cellular changes in the yolk sac associated with death of the fetus occurred between 48–60 hours post injection The villi became shorter thicker and reduced in number the pinocytotic vesicles [illegible]

lets of other cells (Novikoff 63) If he other hand these dense vesicles involved in the process of pinocytosis t fig 1B) they are acting as phago es (Straus 64) and would eventually with lysosomes to form digestive oles

he saccular fuzz lined vesicles are ably pinocytotic in that other studies shown that these vesicles incorporate ker substances gold and thorotrast se 57 Carpenter and Ferm 66) They also be concerned with the process of ytosis for the removal of membrane nants from the cell (text fig 1A) Duve (63) suggested that a mechanism exocytosis might exist but the phe nenon has rarely been observed in a nplex system

## ACKNOWLEDGMENTS

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ies of the resorbing chorio-allantoic placenta have not yet been done but histological preparations indicated that none of the layers of the chorio-allantoic placenta or the decidua basalis responded to Triton treatment as markedly as the yolk sac placenta (Schultz and Schultz, 66)

In resorbing conceptuses the continued distension and vacuolation of the dense and multivesicular bodies was characteristic of the lysosomal area of the yolk sac cells It may be that continued enlargement of these structures was incompatible with the life of the cell In all cases the membrane of the vacuoles appeared to be intact and there was no obvious change in distribution of the acid phosphatase Although acid phosphatase appeared stable during resorption the other acid hydrolases of the lysosomes may have behaved differently

Although the morphology of the vacuoles and the localization of acid phosphatase activity did not change appreciably, drastic changes in structure and presumably function of other organelles occurred during the course of death and resorption of the yolk sac cell These changes loss of the mitochondrial cristae vesiculation of the endoplasmic reticulum decrease in the number and size of the microvilli loss of the pinocytotic vesicles and nuclear pyknosis were definite indications of impending cell death Other studies of cell death have shown that mitochondrial swelling and decay and damage to the endoplasmic reticulum occurred prior to obvious lysosomal degeneration (Weber 63 Dianzani 63)

The survival of the conceptus after Triton treatment was characterized by reorganization of the lysosomal area of the yolk sac cell We cannot say whether the decrease in size of the vacuoles and reaccumulation of the dense material was associated with release of Triton from the cell or was an indication of repackaging of the detergent into smaller vacuoles Survival of the yolk sac cells may not have depended upon reorganization of the lysosomal area since the latter may have occurred as a consequence of the recovery of other cellular systems primarily affected by Triton

The absorptive function of epithelial cell has been well Recently, it has been the yolk sac is also secretory in (Sorokin and Padykula, 64) Both these functions must be related in way to the structures of the distal the cell i e, the microvilli, vesicles, dense bodies and vac s. figure 1 presents the types of in that may occur in this area If the pinocytotic vesicles represent droplets budding off the dense fig 1A) the loss of acid phosphatase tivity from the droplet as it moves tow the apical end of the cell corresponds the loss of the enzyme activity in

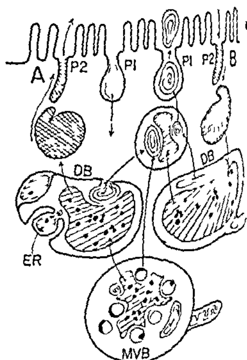
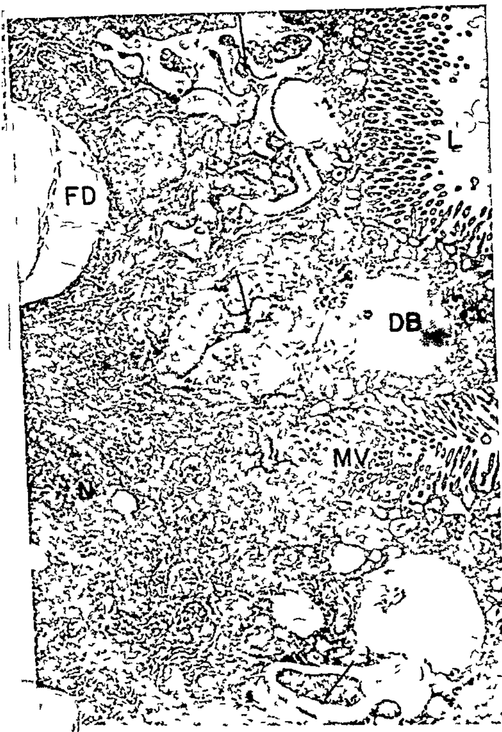



Fig 1 Diagram indicating possible inter tionships of the pinocycoctic vesicles with dense and multivesicular bodies The clear cular pinocytotic vesicles (P1) are presumed serve the process of exocytosis eliminating brane remnants from the yolk sac epithelial The clear vesicles may also result from process of endocytosis Scheme A indicates elimination of a secretory product from the sac cell In this scheme the secretory is formed in the endoplasmic reticulum ( accumulates in the dense bodies (DB) denses as the material moves toward the region of the cell and is eliminated from cell via the dense tubular pinocytotic (P2) Scheme B indicates an alternate way of dense material within the yolk sac (i e endocytosis)


L
FD
DB
MV
N

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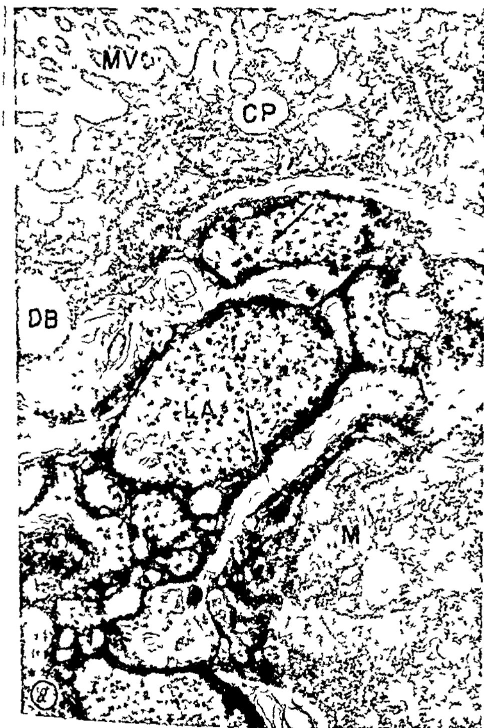
### *Abbreviations*

CP clear pinocytotic vesicle
DB dense body
DP dense pinocytotic vesicles
ER endoplasmic reticulum
FD fat droplet
IS intercellular space
L yolk sac lumen
LA lysosomal area
M mitochondria
MB multivesicular body
MV microvilli
N nucleus
TV vacuole following Triton treatment
V vesiculated region of cell

## PLATE 1

### EXPLANATION OF FIGURE

1 Twelve day control *yolk sac epithelial cells* *Fixative* *Pease s* Acid phosphatase reaction (arrows) 7400 ×


MV
CP
DB
LA
M
8

## PLATE 2

### EXPLANATION OF FIGURE

2 Twelve day control supranuclear region of yolk sac cell Fixative Pease s Acid phosphatase reaction (arrows) 27 000 ×

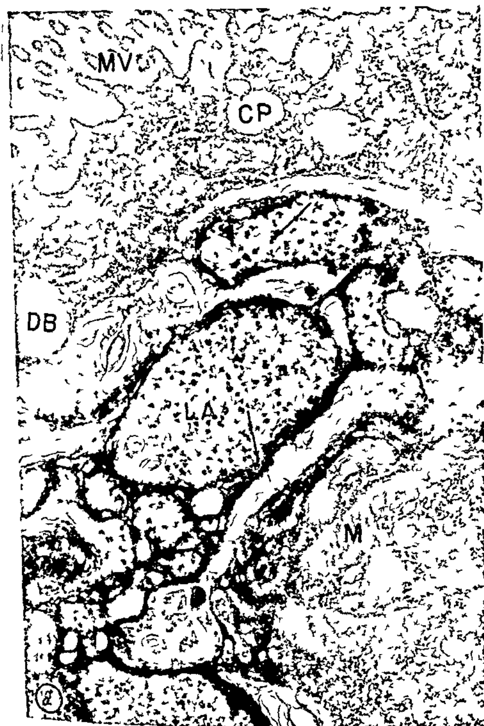
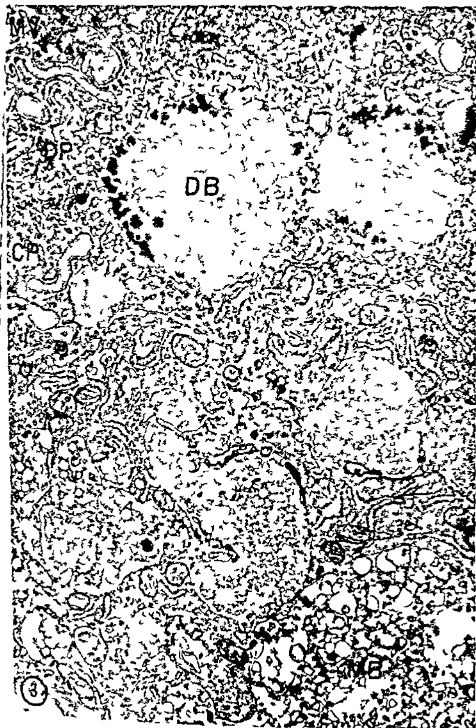

MV
CP
DB
LA
M
12

PLATE 3

EXPLANATION OF FIGURE

3 Twelve day control supranuclear region of cell Fixative Pease s Acid phosphatase reaction inhibited by NaF 15 000 ×


MV
DP
DB
CP
MB
3

## PLATE 4

### EXPLANATION OF FIGURES

4 Twelve day control example of fusion of mitochondria with multi vesicular body Fixative glutaraldehyde 45 000 ×

5 Twelve day control Fixative Pease s Acid phosphatase reaction (arrows) 18 000 ×

MB

MB

## PLATE 4

### EXPLANATION OF FIGURES

4 Twelve day control example of fusion of mitochondria with "multi vesicular body Fixative glutaraldehyde 45 000 ×

5 Twelve-day control Fixative Pease s Acid phosphatase reaction (*arrows*) *18 000* ×

## PLATE 5

### EXPLANATION OF FIGURES

6 Fifteen day control supranuclear region of yolk sac cell Pease s fixative Acid phosphatase reaction (arrow) 32 000 ×

7 Twenty-day control supranuclear region of yolk sac cell Holt s fixative Acid phosphatase reaction (arrow) 15 000 ×

DB
ER
5



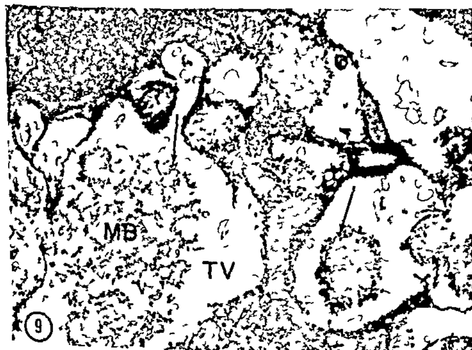
## PLATE 6

### EXPLANATION OF FIGURES

8 Twenty-day control apical area of yolk sac cell Holt s fixative 27 000 ×

9 Four hour Triton treated (200 mg) yolk sac cell day 12 of pregnancy Pease s fixative Acid phosphatase reaction (arrows) 15 000 ×

MV
CP
8



## PLATE 7

### EXPLANATION OF FIGURE

10 Thirteen day Triton treated (300 mg) yolk sac cells 48 hours after injection Pease's fixative Acid phosphatase reaction (arrows) 6500 ×


L
MV
TV
10

## PLATE 7

### EXPLANATION OF FIGURE

10 Thirteen day Triton treated (300 mg) yolk sac cells 48 hours after injection *Pease's fixative* Acid phosphatase reaction (arrows) 6500 ×


DB
T.V
11

## PLATE 8

### EXPLANATION OF FIGURE

11 Thirteen day Triton treated (300 mg) yolk sac cells 48 hours after injection Pease s *fixative* Acid phosphatase reaction (arrows) 20 000 ×


DB
T.V
11

## PLATE 9

### EXPLANATION OF FIGURE

12 Thirteen day Triton treated (200 mg) yolk sac cells 48 hours after injection Example of blebbing of endoplasmic reticulum into vacuoles (arrows) Pease s fixative Acid phosphatase reaction re action inhibited with NaF 14 000 ×

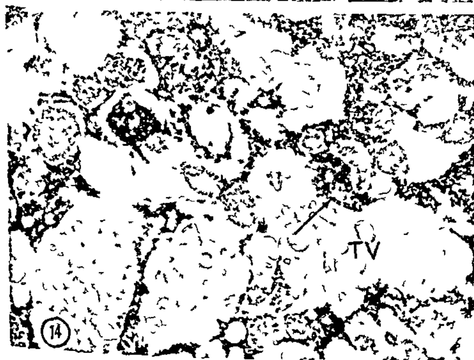

MV
TV
12

## PLATE 9

### EXPLANATION OF FIGURE

12 Thirteen day Triton treated (200 mg) yolk sac cells 48 hours after injection Example of blebbing of endoplasmic reticulum into vacuoles (arrows) Pease's fixative Acid phosphatase reaction reaction inhibited with NaF 14 000 ×


DB

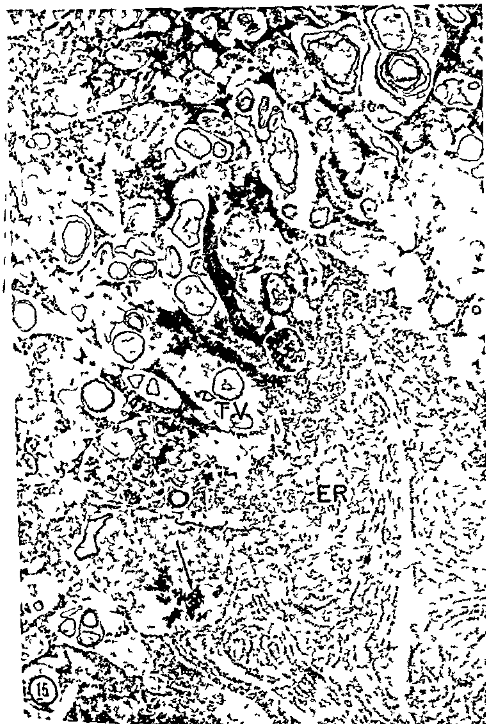

TV
14

## PLATE 10

### EXPLANATION OF FIGURES

13 Fifteen day Triton treated (200 mg) yolk sac epithelial cell 96 hours after injection Viable fetuses at term Pease s fixative Acid phosphatase reaction (arrow) 13 000 ×

14 Sixteen-day Triton treated (300 mg) yolk sac cell 120 hours after injection Viable *fetuses* at term Pease s fixative Acid phosphatase reaction (arrow) 10 000 ×


TV
ER
15

## PLATE 11

### EXPLANATION OF FIGURE

15 Twenty day Triton treated (300 mg) yolk sac cell nine days after Triton injection *Viable fetuses* Holt's fixative Acid phosphatase reaction (arrow) 12 000 ×


N
IS
TV
V
L
MV
V
IS
16

## PLATE 12

### EXPLANATION OF FIGURE

16 Fourteen day Triton treated (300 mg) yolk sac cell 72 hours after injection Five dying yolk sac cells from animal that resorbed litter before term Pease s fixative Acid phosphatase reaction (arrow) 7000 ×


TV
M
N
11

## PLATE 13

### EXPLANATION OF FIGURE

17 Fourteen day Triton treated (200 mg) yolk sac cell 72 hours after injection Litter resorbed Pease's fixative Acid phosphatase reaction (arrow) 15 000 ×

## PLATE 14

### EXPLANATION OF FIGURES

18 Fourteen day Triton treated (200 mg) yolk sac cell apical region Litter resorbed Pease s fixative Acid phosphatase reaction (arrow) 27 000 ×

19 Fourteen-day Triton treated (200 mg) yolk sac cell apical region Litter resorbed Pease s fixative Acid phosphatase reaction (arrow) 17 500 ×

ıistochemıcal Study of the Esterases
.he Rat Heart [1]

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*ABSTRACT* The histochemistry of esterases was studied in the rat heart using various methods of tissue preparation and a large number of substrates inhibitors and activators

Non-specific esterase and cholinesterase was demonstrated in the fibers of the atrioventricular conduction system in atrial and ventricular muscle fibers in cardiac neurons and in some of the nerve fibers of the heart The highest concentration of both types of enzymes was found in the conduction system and in the neural elements Pericytes and macrophages showed only non specific esterase activity

Two broad types of non specific esterase activity cytoplasmic and lysosomal were distinguished in the pericytes macrophages and cardiac muscle fibers by the use of selective inhibitors and activators The cytoplasmic activity is due to the presence of B and C esterases and was better demonstrated with esters of α naphthol than with naphthol AS derivatives or indigogenic esters The esterase activity of lysosomes appears to be due to a group of fluoride- and organophosphate resistant en zymes some of which are probably cathepsins

Although the histochemical localızatıon cholınesterase and thiolacetic acid ester in cardiac muscle recently has been ıdied by electron mıcroscopy (Barrnett d Palade 59 Karnovsky and Hug 63 ırnovsky 64) the general topography of e various esterases of the rat heart has en the subject of relatively few investi ıtions Nachlas and Seligman (49) omori (52) and Chessick (53) de rıbed unidentified cells in the rat heart ıat reacted with α naphthyl acetate in ıraffin sections of acetone fixed tissues ıe remainder of the myocardıum showed o activity Ravin Zacks and Seligman 53) reported the hydrolysis of β-naphthyl cetate in unfixed frozen sections of rat eart with the formation of a diffuse ranular precipitate ın the muscle fibers arbonell (56) found α naphthyl acetate sterase activity in ınterstitial cells be ieved to be macrophages scattered hroughout the myocardium the atrio- entricular conduction system (AVCS) re ıcted strongly with choline esters but not with α naphthyl acetate More recently Joo and Csillik (62) applied the thiolace tic acid and the indoxyl acetate methods to sections pretreated with hyaluronidase and found esterase activity in the cytoplasm and intercalated discs of the rat heart

Significant advances ın the histochemi cal methodology of esterases (Pearse 61a Burstone 62 Barka and Anderson 63) have been made since the publication of some of the preceding histochemical re ports and the present investigation ap- peared justified by the fact that these improvements have not been applied sys tematically to the study of the heart

## MATERIAL AND METHODS

### *Fixation*

Male albino rats weighing from 250 to 300 gm were used In some experiments the hearts were removed rapidly after de capitation and frozen ın dry ice isopen tane fresh frozen sections were then cut in a cryostat and post fixed for periods of time varying from 30 minutes to 16 hours ın one of the following fixatives 4% neu tralized formaldehyde containing 1% cal cium chloride (formol-calcium) with and without the addition of 0 44 M sucrose 4% formaldehyde ın 0 1 M phosphate buf fer 7 4 4% glutaraldehyde in 0 1 M phos phate buffer pH 7 4 and 4% glutaralde- hyde in 0 1 M cacodylate buffer pH 7 2 In other experiments the heart was per

[1] S pport d by research grant HE-06100-04 and by T ining gr nt 5T1 GM 793 from the Unit d States Public He lth Servi e

## RESULTS

terase activity was demonstrated in ollowing structures of the heart

The cytoplasm of the specialized :le cells of the atrioventricular node the bundle of His and its two main ches (figs 1–4 23 25–28 31 32)

The cytoplasm and lysosomes of the l and ventricular muscle cells (figs ! 17–20 23 24 31 32)

The neural elements including the ganglia and their postganglionic e fibers the nervous plexuses sur ding the atrioventricular node and bundle of His and some of the nerve s in the atria ventricles and atrio- ricular ring (figs 2 3 6 24 28–30)

The cytoplasm and lysosomes of vari types of interstitial and pericapillary present throughout the myocardium epicardium in the mural and valvular ocardium and in the adventitia of the e blood vessels at the base of the heart s 1–4 7–17 19–22)

he intensity of the reaction at the vari enzymatic loci the extent of diffusion the quality of the morphological pres ation of tissues varied according to the hod of fixation For the study of non cific esterases pre fixation for two rs with 4% glutaraldehyde in 0 1 M odylate buffer pH 7 2 gave the best r all results particularly when pre ed by in situ perfusion of the heart h the fixative Higher enzymatic activ and greater evenness of fixation were nonstrated after this procedure than ter fixation by immersion overnight in rmol-calcium which otherwise gave ex llent results Less satisfactory morphol y much more diffusion and consider bly less enzymatic activity were found ith all post fixation procedures Of the tter fixation in the cold for 30 minutes ith formol-calcium gave the most accept ble results Prolongation of the time of ost fixation resulted in gradual reduction f the esterase activity of the muscle fi ers which showed only a slight reaction fter 12 hours of post fixation

Variable amounts of non-specific ester ses are known to be extracted from fresh rozen sections during the process of post ixation and during exposure to the solu ions of buffers and inhibitors (Nachlas et al 56 Hannibal and Nachlas 59) For this reason the studies on the effects of enzymatic inhibitors and activators were made on free floating sections from pre fixed tissues in which such losses are minimal

Cholinesterases were best studied in fresh frozen sections briefly fixed in cold buffered formaldehyde since the enzyma tic activity was strongly inhibited by pro- longed formaldehyde fixation and by the glutaraldehyde mixtures

As shown in table 1 the patterns of histochemical staining observed in each of the esterase active sites depended pri marily on the chemical structures of the substrates employed for the reaction and our results will be described accordingly

*Esters of α naphthol* The strongest staining was produced by α naphthyl ace tate Progressively less intense staining was observed with the propionate and butyrate esters and only a faint reaction was given by α naphthyl valerate No stain ing was noted with α naphthyl caprylate laurate or myristate Of the two types of coupling agents best results were ob tained with hexazotized p-rosanilin which produced much better defined localization than Diazo Blue B

A moderately intense reaction was ob- tained in the cytoplasm of the specialized muscle cells of the atrioventricular node and the bundle of His and its two main branches (figs 1–4) The esterase activ ity of the peripheral ramifications of the AVCS was similar to that of ventricular myocardial cells Atrial muscle fibers re acted less intensely than the AVCS but more strongly than ventricular muscle Two distinct types of enzymatic activity towards esters of α naphthol were distin guished in the muscle fibers by the use of selective inhibitors (table 2) One was responsible for the diffuse cytoplasmic staining (fig 5) the other was localized in sarcoplasmic bodies that were identi fied as lysosomes on the basis of electron microscopy and staining for acid phospha tase No staining was observed in the intercalated discs The diffuse cytoplasmic esterase activity which is sensitive to pro- longed aldehyde fixation was completely inhibited by $10^{-8}$ M DFP (diisopropylfluo- rophosphate) $10^{-5}$ M $E_{600}$ (diethyl p-

fused *in situ* for 15 minutes with one of the fixatives listed above and then subjected to further fixation with the same solution for two hours in the glutaraldehyde mixtures and overnight in the formaldehyde solutions Specific areas of the heart were then dissected washed in several changes of buffer or 0 44 M sucrose frozen rapidly and sectioned in a cryostat

For the study of the atrioventricular conduction system it became necessary to use post fixed frozen sections cut serially since considerable difficulty was often encountered in handling free floating prefixed sections of the area of the atria and the atrioventricular ring

*Histochemical staining*

*Seven groups of esterase substrates were used*

1 *The following α naphthyl esters of straight chain aliphatic acids were employed Acetate propionate butyrate valerate caprylate laurate and myristate* Two types of agents were utilized to couple the liberated α naphthol stabilized diazonium salts and hexazotized p rosanilin In both cases the incubation was performed at a pH ranging from 6 5 to 8 2 Since best results were obtained at pH 7 0 this was used throughout the study For the hexazotized p rosanilin the method of Barka and Anderson (63) was followed and sections were incubated at room temperature for 15 minutes Of the diazonium salts used (Diazo Blue B Diazo Blue RR Diazo Blue BB Diazo Red RC and Fast Violet B) best results were obtained with Diazo Blue B which was used in all subsequent experiments Sections were incubated at room temperature for ten minutes in a medium prepared according to the method of Pearse (61b)

2 *Napthol AS esters* Naphthol AS D acetate naphthol AS D chloroacetate naphthol AS LC acetate and naphthol AS BG acetate were used Sections were incubated for 30 minutes at room temperature according to the method of Burstone (62) The double staining technique of Shnitka and Seligman (61) was followed for the differential demonstration of cytoplasmic and lysosomal esterase activity in the same tissue section

3 *Indigogenic substrates* The technique of Holt and Withers (58) was followed using indoxyl acetate 5-bromoindoxyl acetate and 5 bromo-4-chloroindoxyl acetate as substrates In some experiments the ferricyanide-ferrocyanide redox system was used in reduced concentrations (0 0005 M instead of 0 005 M) Sections were incubated for 30 minutes at 37°C Incubation times were prolonged to 1[illegible] hours in media containing high concentrations of the redox system

4 *Choline esters of long-chain fatty acids* The modified Gomori technique of Barka and Anderson (63) was used Sections were incubated for two hours at 37°C in media containing either lauroyl or myristoyl choline as substrate

5 *Thiocholine esters* The procedure of Koelle and Friedenwald (49) was followed using acetyl and butyrylthiocholine sections were incubated for two hours at 37°C

6 *Thiolacetic acid* The method of Wachstein Meisel and Falcon (61) was used sections were incubated at room temperature for 15–30 minutes

7 *Tweens* The method of Gomori (45) was followed using Tween 80 Sections were incubated with and without the addition of $10^{-3}$ M sodium taurocholate at 37°C for periods of time ranging from 30 minutes to 16 hours

Control procedures included the incubation of heat inactivated sections and the use of media that contained all reagents except the substrate

The effect of the enzymatic activators and inhibitors listed in table 2 was studied In these experiments the sections were pre incubated for one hour at room temperature in a buffered solution containing the inhibitor after which they were transferred to a complete incubating medium and processed in the same manner as sections that had been (a) untreated and (b) treated with the buffered solution without the inhibitor Whenever possible (i e when it did not react with any of the components of the medium) the inhibitor was also added to the final incubating medium

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A moderately intense reaction was obtained in the cytoplasm of the specialized muscle cells of the atrioventricular node and the bundle of His and its two main branches (figs 1–4) The esterase activity of the peripheral ramifications of the AVCS was similar to that of ventricular myocardial cells Atrial muscle fibers reacted less intensely than the AVCS but more strongly than ventricular muscle Two distinct types of enzymatic activity towards esters of α naphthol were distinguished in the muscle fibers by the use of selective inhibitors (table 2) One was responsible for the diffuse cytoplasmic staining (fig 5) the other was localized in sarcoplasmic bodies that were identified as lysosomes on the basis of electron microscopy and staining for acid phosphatase No staining was observed in the intercalated discs The diffuse cytoplasmic esterase activity which is sensitive to prolonged aldehyde fixation was completely inhibited by 10 M DFP (diisopropylfluorophosphate) $10^{-5}$ M E₆₀₀ (diethyl p-

TABLE 1

*Distribution of esterase activity in rat heart as shown by different types of histochemical substrates Tissues were pre fixed for two hours in cacodylate buffered gultaraldehyde for the experiments in which naphtholic and indigogenic substrates and thiolacetic acid were used Post fixation for 30 minutes in cold formol-calcium was used for the study of cholinesterase substrates The intensity of staining is expressed in a scale from 0 to 4+ in reference to the structure showing the highest activity with each substrate*

| Incubating medium | Cardiac muscle cells | | | Iterstitial cells | | Neural elements | Mast cells |
|---|---|---|---|---|---|---|---|
| | Atrial | AVCS | Ventric | Cytoplasm | Lysosomes | | |
| a Naphthol acetate hexazotized p-rosanilin | ++ | +++ | + | ++++ | ++++ | +++ | + |
| Naphthol AS-D acetate Fast Garnet GBC salt | + | ++ | + | +++ | ++++ | +++ | +++ |
| Indigogenic substrates with 0 0005 M redox system | ++ | +++ | + | +++ | ++++ | +++ | ± |
| Thiolacetic acid | + | ++ | + | ± | ++++ | ++ | 0 |
| Lauroyl choline | ++ | +++ | ± | 0 | 0 | ++++ | 0 |
| Myristoyl choline | + | +++ | ± | 0 | 0 | ++++ | 0 |
| Acetylthiocholine iodide | ++ | +++ | + | 0 | 0 | ++++ | 0 |
| Butyrylthiocholine iodide | ++ | +++ | + | 0 | 0 | ++++ | 0 |
| Tween 80 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |

nitrophenylphosphate) 10⁻³ M merc… acetate and by 0 005 M concentra… of the ferricyanide ferrocyanide redox system of Holt It was slightly inhibited by 10⁻⁴ M copper sulfate 10⁻³ M silver nitrate and was unaffected by 10⁻³ M iso-OMPA (tetra-isopropylpyrophosphoramide) 10⁻³ M … phenylpropionic acid 10⁻³ M sodium … rocholate 10⁻³ M lead nitrate 10⁻³ M sodium iodoacetate and by 0 1 M N-ethyl maleimide The diffuse cytoplasmic esterase activity was activated by 10⁻⁴ M cysteine No activation could be demonstrated with 10⁻⁴ M PCMB (p-chloromercuribenzoate) except after treatment with 10⁻³ M E₆₀₀ which irreversibly inhibited all the diffuse cytoplasmic esterase activity

The small number of lysosomes present in the cardiac muscle cells particularly in the perinuclear area contained a fluoride and organophosphate resistant type of esterase activity similar to that of the lysosomes of the interstitial cells In view of the fact that the localization of this type of esterase activity was restricted to lysosomes the term lysosomal esterase will be used throughout this paper in reference to the fluoride and E₆₀₀ resistant esterase activity present in lysosomes Similarly the fluoride and E₆₀₀ sensitive esterase activity responsible for the diffuse cytoplasmic staining will be referred to as cytoplasmic esterase

Various types of interstitial cells present throughout the heart (figs 7–17) gave an intense esterase reaction Many of these cells were pericytes (figs 9–1…) which had numerous elongated cytoplasmic processes and were closely related to the walls of the capillaries and the s… endocardium and subepicardium (fig 13) others mostly macrophages and fibroblasts were unrelated to small blood vessels and were especially prominent in the upper portion of the interventricular septum in the atrioventricular ring in the adventitia of the great vessels and in the stroma of the cardiac valves (figs 14–16) The cytoplasmic processes of these cells were best demonstrated in sections from pre fixed tissue blocks Mast cells gave only a faint reaction with the a naphthol substrates

TABLE 2

*Effect of inhibitors and activators on the hydrolysis of naphtholic substrates by rat myocardium pre fixed in cacodylate buffered glutaraldehyde*

| Inhibitors o activator | Cytoplasm of mu cle fibers | Iterstitial c ll | |
|---|---|---|---|
| | | Cytoplasm | Lysosome |
| serine 10 M | I+ | 0 | 0 |
| DFP 10 M | I++++ | I++++ | I++ |
| $E_{600}$ 10 M | I++++ | I++++ | I++ |
| $E_{600}$ 10 M | I++++ | I++++ | 0 |
| so-OMPA 10 M | 0 | 0 | 0 |
| odium fluoride 10 M | I++++ | I++++ | 0 |
| Mercuric acetate 10 M | I++++ | I++++ | I+++ |
| opper sulfate 10 M | I++ | I++ | I+ |
| Silver nitrate 10 M | I++ | I++ | I+ |
| Lead nitrate 10 M | 0 | 0 | 0 |
| β Phenylpropionic acid 10 M | 0 | 0 | 0 |
| Cysteine 10 | A+++ | A+++ | A+ |
| Taurocholate 10 M | 0 | 0 | 0 |
| Sodium iodoacetate 10 M | 0 | 0 | 0 |
| N-ethylmaleimide 0 1 M | 0 | 0 | 0 |
| PCMB 10 M | 0 | 0 | 0 |
| Inhibition by $E_{600}$ 10 M followed by PCMB $10^{-4}$ M | 0 | 0 | 0 |
| Inhibition by $E_{600}$ 10 M followed by PMCB 10 M | A++ | A+ | A± |

A ti ti n i d gn ted by A and inhibition by I. + 25% ++ 50% +++ 75% ++++ 100% 0 n effe t.

As in the muscle fibers two types of rase cytoplasmic and lysosomal were monstrated in the interstitial cells (table The cytoplasmic enzyme (figs 9 10) s less sensitive to aldehyde fixation an that of the muscle fibers and was mpletely inhibited by organophosphates M sodium fluoride (fig 11) $10^{-3}$ M rcuric acetate and by 0 005 M concen tion of the ferricyanide ferrocyanide re x system of Holt It was partially in bited by silver nitrate and by 10 M pper sulfate but was not affected by ) M iso-OMPA 10 M eserine 10 M phenylpropionic acid 10 M lead ni ate 10 M iodoacetate 10 M sodium urocholate or by 0 1 M N-ethylmaleim le The enzyme was activated by cys ine however the cytoplasmic esterase f the interstitial cells in contrast to that f the muscle fibers was only slightly ctivated by 10 M PCMB after inhibition y 10 M $E_{600}$

Many of the interstitial cells also con ained lysosomal esterase activity which vas best demonstrated in sections in which the other types of esterase had been nactivated either by fixation or by the use selective inhibitors (fig 11) These ex showed that there was consider able variation in the number of lysosomes present in the various types of interstitial cells The lysosomal esterase was promi nent in macrophages the cytoplasmic en zyme accounted for the major part of the esterase activity of the pericytes which had few lysosomes

*Naphthol AS esters* In general the staining pattern obtained with these sub strates was qualitatively similar to that given by the esters of α naphthol (table 1) The best localization was obtained with naphthol AS D acetate which produced intense staining of the interstitial cells and neural elements but only a very weak re action in the cytoplasm of the muscle cells The cytoplasmic processes of the intersti tial cells were not as well delineated by the naphthol AS substrates (fig 12) as by the α naphthyl esters This was at least partly due to the granular character of the dye deposits produced by the naphthol AS com pounds Mast cells reacted much more intensely with naphthol AS esters than with the α naphthyl substrates Their re activity toward naphthol AS D acetate was similar to that of the interstitial cells how ever naphthol AS D chloroacetate a sub strate that gave only a faint reaction in all other myocardial components was hydro-

lyzed by the mast cells at a much faster rate than by the interstitial cells Studies on the influence of enzymatic inhibitors upon the hydrolysis of naphthol AS sub strates gave results similar to those ob tained with the esters of α naphthol

The double staining technique of Shnitka and Seligman (61) based on the reversible inhibition of the cytoplasmic esterase by fluoride was used to show his tochemically the presence of both types of esterase i e, lysosomal and cytoplasmic in the interstitial cells An attempt was made to demonstrate the cytoplasmic esterase by using various naphtholic sub strates after staining for the lysosomal esterase by the indigogenic method of Holt followed by prolonged washing to remove the ferricyanide ferrocyanide redox sys tem However it was found that as shown by Shnitka and Seligman (61) the in hibition of the cytoplasmic esterase by the redox system is to a large extent irre versible

*Indigogenic esters* Best results were obtained with 5 bromo 4 chloroindoxyl ace tate Only an extremely faint reaction was observed in the muscle fibers even after long periods of incubation when Holt s complete indigogenic medium was used For this reason several changes were made in the composition of the incubating me dium the results obtained are summarized in table 3 With Holt s complete medium esterase activity was demonstrated in the AVCS in neurons and nerves of the car diac ganglia and in the lysosomes of the cardiac muscle fibers and interstitial cells The cytoplasmic esterase of the interstitial and muscle cells was inhibited by the high pH and by the 0 005 M concentration of the ferricyanide ferrocyanide redox sys tem (fig 17) Histochemical localization similar to that produced by the naphthol AS esters was observed (figs 18 20 23 24) when the final concentration of the redox buffer was reduced to 0 0005 M and or the pH of the incubating solution was changed from 8 5 to 7 0 Omission of the 1 M so dium chloride or its replacement by su crose also resulted in a modest increase in the activity of the cytoplasmic esterase *Even under apparently optimal conditions for the reaction* the staining of the inter stitial cells was less well defined than that seen with the naphtholic substrates and the number of cells stained by the indigo methods appeared smaller At pH 7 0 and using the 0 0005 M concentration of the redox system the effect of the various in hibitors tested (figs 19 21) was similar to that found with the naphtholic sub strates

*Choline esters* The results obtained with these esters (figs 25–32) differed from those of the other substrates em ployed in this study (table 1) With lauroyl and myristoyl choline a positive reaction was observed in some of the nerve fibers and in the cytoplasm of the neurons and specialized muscle fibers of the bundle of His (figs 25–28) Staining of the cardiac muscle fibers however faint and diffuse was obtained with lauroyl choline but not with myristoyl choline The interstitial

TABLE 3

*Effect of various modifications made in Holt s indigogenic medium on the intensity of the esterase reaction of rat myocardium pre fixed in cacodylate buffered glutaraldehyde The relative intensity of staining is expressed in a scale from 0 to 4+*

| Composition of the medium | Cardiac muscle fibers | Iterstitial cells | | Neural elements |
|---|---|---|---|---|
| | | Cytoplasm | Lysosomes | |
| Complete Holt s medium | ± | ± | ++++ | + |
| Concentration of ferricyanide ferrocyanide redox system reduced from 0 005 M to 0 0005 M | ++ | +++ | ++++ | +++ |
| Complete Holt s medium pH reduced from 8 5 to 7 0 | + | + | ++++ | ++ |
| Complete Holt s medium NaCl ommitted | + | + | ++++ | +++ |

and mast cells did not react with any e esters of choline or thiocholine ityrylthiocholine was hydrolyzed more lly than was the acetyl ester both of compounds were hydrolyzed at a h faster rate than the long chain cho esters In both instances the fibers in bundle of His gave a more intense re on than the atrial fibers the latter re d more strongly than the ventricular s (figs 31 32)

The hydrolysis of all choline esters (table was completely inhibited in all sites by M eserine 10⁻ M E₆₀₀ and by 10⁻ M moderate inhibition was produced 10 M iso-OMPA (figs 29 30) and ked inhibition by 10 M sodium fluo Taurocholate had no effect on the tion

*hiolacetic acid* Using fresh frozen fixed sections thiolacetic acid re ed in moderate diffuse staining of the plasm of the muscle fibers and neural nents the cytoplasm of the interstitial s failed to stain Staining in the spe lized muscle fibers of the bundle of His more intense than in the atrial mus fibers the latter gave a stronger reac than the ventricular muscle fibers is reaction was little affected by 10 M rine but was totally inhibited by 10 M

staining of tissues pre fixed with glutar lehyde revealed primarily the lysosomes the interstitial cells (fig 22) which ld not be clearly distinguished in the st fixed sections Staining of the cyto ism of the muscle cells was faint and no reaction could be detected in the cytoplasm of the interstitial cells

## DISCUSSION

Esterases are a group of enzymes that hydrolyze the carboxylic acid esters of mono- and polyhydric alcohols and phe nols (Gomori 52 Gomori and Chessick 53) They possess a low degree of substrate specificity and occur in a wide variety of molecular forms (isozymes) with broadly overlapping enzyme-substrate characteristics (Markert and Hunter 59 Hunter and Burstone 60) Esterases may be classified according to the following scheme based on that of Pearse (61a)

1 *Non specific esterases* Their activ ity is not affected by eserine According to their sensitivity to other inhibitors and ac tivators they can be classified into

a A-esterases (arom-esterases organo phosphate resistant type I esterases) which are completely inhibited by $10^{-2}$ M E₆₀₀ partially inhibited by 10⁻ M PCMB and activated by cysteine Their activity is also inhibited by heavy metal ions such as $Hg^{+}$ $Ni^{+}$ $Pb^{++}$ and by N-ethylmalei mide

b B esterases (aliesterases organophos phate sensitive esterases) which are com pletely inhibited by 10 M E₆₀₀ they are not affected by heavy metal ions and are activated by cysteine Sodium fluoride in a concentration of 10⁻ M is a potent in hibitor of the activity of this type of ester ases (Shnitka and Seligman 61)

c C-esterases (organophosphate resist ant type II esterases cathepsin like ester

TABLE 4

*The effect of enzymatic inhibitors on the hydrolysis of choline esters by sections of rat heart post fixed fo 30 minutes in cold formol-calcium*

| Inhibito | Substr te | Myocardial fibers | B ndl of His | Neural elements |
|---|---|---|---|---|
| DFP 10 M | All choline esters | I++++ | I++++ | I++++ |
| E₆₀₀ 10 M | All choline esters | I++++ | I++++ | I++++ |
| Eserine 10 M | All choline esters | I++++ | I++++ | I++++ |
| Iso-OMPA 10 M | Acetylthiocholine | I+++ | I+ | I+ |
| | Butylrylthiocholine | I+++ | I++ | I++ |
| | Long-chain choline esters | I+ | I+ | I+ |

I = inhibition xpressed on a sc le from 0 to 4+

ases), which are inhibited by $10^{-3}$ M β phenylpropionic acid and activated by PCMB They resemble A esterases in their resistance to organophosphates and in their sensitivity to heavy metal ions and N-ethyl maleimide

2 *Cholinesterases* Their activity is completely inhibited by $10^{-5}$ M eserine $10^{-5}$ M DFP, and by $10^{-7}$ M $E_{600}$ They are subdivided into

a true cholinesterases (specific cholin esterases acetylcholinesterases) which are not affected by $10^{-5}$ M iso-OMPA and

b pseudocholinesterases which are selectively inhibited by $10^{-5}$ M iso OMPA

3 Lipases, which are activated by sodium taurocholate and preferentially hydrolyze esters of aliphatic fatty acids with a straight carbon chain that contains more than 12 carbon atoms

The capacity of these enzymes to act upon a number of different substrates renders their characterization very difficult Furthermore the use of histochemical substrates permits only a limited characterization of the esterases of the heart since as shown by electrophoretic experiments (Hunter and Burstone 60 Barron et al 63), histochemical reactions demonstrate the total activity of a family of isozymes that are capable of hydrolyzing a wide variety of substrates Thus thiolacetic acid (Crévier and Belanger 55 Wachstein Meisel and Falcon 61) and the naphtholic and indigogenic substrates (Hobbiger 57 Pearse 61a) are attacked by cholinesterases and by various types of non specific esterases However the hydrolysis of choline esters by rat tissues is considered to be exclusively due to true and pseudocholinesterases (Aldridge 54 Allen et al 58 Hobbiger 57)

For these reasons the use of selective inhibitors is necessary for the classification of esterases (Aldridge 54 Bayliss and Todrick 56 Pearse 61a Burstone 62) and several distinctive features emerge from a comparison of the effects of inhibitors and activators on the esterase activity present in the various components of the rat heart

*The atrioventricular conduction system* Non specific esterase and cholinesterase activity were demonstrated histochemically in the specialized muscle fibers of the atrioventricular node and bundle of [illegible] and its two main branches but not in the peripheral ramifications both types of enzymes were also present in the cardi[illegible] ganglia and in the nervous plexuses [illegible]rounding the AVCS

Non specific esterase activity has [illegible] been detected previously in the AVCS of the rat but is known to occur in that of other mammalian species (Carbonell 5[illegible] The negative reaction towards naphth[illegible] substrates observed by Carbonell (56) [illegible] the AVCS of the rat was probably d[illegible] the inhibition of the enzymatic activity b[illegible] prolonged fixation and by the use of [illegible] cubating media adjusted at a high pH Ca[illegible]bonell (56) reported the presence of ch[illegible]linesterase in the fibers of the AVCS of the rat and other workers have made sim[illegible]lar observations in other species (Gom[illegible] 48, Gerebtzoff, 53) This enzyme has been studied in the nervous plexuses of the AVCS of other mammals ([illegible] Kakueva, 58, Robb 65) but not in the [illegible] of the rat

The enzymatic activity of the mus[illegible] fibers of the AVCS against naphtholic a[illegible] indigogenic substrates appeared to b[illegible] caused by the presence of both non speci[illegible] esterase and cholinesterase This w[illegible] shown by the partial inhibition of the h[illegible]drolysis of these substrates by eser[illegible] which inhibits both true and pseudoch[illegible] esterases but has no influence upon n[illegible] specific esterases (Pearse 61a) I[illegible] OMPA which is known to inhibit al[illegible] completely the pseudocholinesterases b[illegible] not the true cholinesterases of rat tis[illegible] (Bayliss and Todrick 56) had little [illegible]fect on the hydrolysis of naphtholic a[illegible] choline esters by the AVCS and surrou[illegible]ing nerve fibers However eserine [illegible]hibited completely the hydrolysis of c[illegible] line esters by these structures wh[illegible] indicated that the major part of their c[illegible]linesterase activity is of the true cholin[illegible]terase type

*Cardiac ganglia* The neurons of [illegible] cardiac ganglia and their post gangl[illegible] nerve fibers were intensely stained [illegible] cholinesterase and non specific ester[illegible] Most of their cholinesterase activity w[illegible] of the true type which conforms to [illegible] histochemical pattern of other paras[illegible]

:rtic ganglia (Koelle 62) and agrees . the findings of Gerebtzoff (53) on cardiac ganglia of other species

*ie atrial and ventricular muscle fibers* ase activity was found in the lyso-s and in the cytoplasm of the cardiac le fibers The lysosomal esterase was onstrated only in sections from fixed e blocks presumably because of the iption of the lysosomes during freez and thawing of unfixed tissues The somes of the cardiac muscle fibers very small in size and number and r esterase activity appeared similar to of the lysosomes of other tissues lt 63)

he cytoplasmic esterase which is sen e to the effects of fixatives was best wn in sections of glutaraldehyde-caco-ite fixed tissues As recognized by nn Zacks and Seligman (53) the fail of some investigators to detect this en ne was caused by its inactivation during longed fixation Subsequent investiga is (Shnitka and Seligman 61) have efully documented the fact that the oplasmic esterase of liver and kidney ls is inhibited by prolonged formal iyde fixation The negative results ained by Pearson and Defendi (57) us Holt s indigogenic method on rat myo-dium fixed for a short period of time y be explained by their use of a rela ely high concentration of the ferricya le ferrocyanide redox system which is w known to produce complete and par lly irreversible inhibition of the cyto-ismic esterase of liver and kidney hnitka and Seligman 61) and as own in this study of cardiac muscle The hydolysis of choline esters by the toplasm of the muscle fibers was com itely inhibited by eserine However ine produced only minimal inhibition the hydrolysis of naphtholic and indigo-nic esters by the muscle fibers These ta and the marked inhibition by iso-OMPA of the reaction of the muscle fibers against choline esters suggest that both non specific esterase and pseudocholines erase are present in the cytoplasm of the ac muscle cells

The intensity of staining obtained with naphtholic and choline esters was greater in the atria than in the ventricles These results agree with the biochemical studies of Ord and Thompson (50) which showed that in the rat the cholinesterase activity of atrial tissue is three times higher than that of ventricular muscle Our observa tions on the relationship of the relative rates of hydrolysis of various α naphtholic esters by the muscle fibers and interstitial cells to the length of the carbon chain of the esterified acids are also in agreement with biochemical measurements (Hug gins and Moulton 48) of the rates of the *in vitro* hydrolysis of a series of p-nitro-phenyl esters by rat myocardial and testic ular esterases

The presence of lipase activity has been observed histochemically in the myocar dium of several mammals but not in the rat (George and Iype 60) However the negative reaction obtained with Tween 80 in the presence and absence of taurocho-late tends to exclude the possibility that the histochemically detectable esterase ac tivity of the muscle fibers of the rat heart is caused by lipase

The use of selective inhibitors revealed that the major part of the non specific esterase activity of the cardiac muscle fibers conforms to the criteria of a B ester ase i e an organophosphate sensitive esterase similar to that present in the cyto-plasm of other cell types (Wachstein Meisel and Falcon 61 Shnitka and Selig man 61) After treatment with 10 M $E_{600}$ no esterase activity could be detected in the cytoplasm of the muscle fibers how ever when sections exposed to this inhibi tor were further treated with 10 M PCMB a small but definite activation of the re action was noted PCMB is used to classify the types of organophosphate resistant esterases since it activates C esterase and inhibits A esterase (Pearse 61a) There fore the preceding data were interpreted as indicating that in addition to the B type of esterase the muscle fibers also contain a small amount of C esterase the activation of which could not be histo-chemically detected unless other types of esterase were simultaneously inhibited The presence of B and C esterases in rat ventricular muscle fibers has been recog nized by Karnovsky and Hug (63) in their electron microscopic study of thiolacetic acid esterase As pointed out by these

authors it is unusual to find an organophosphate resistant aldehyde sensitive esterase and the significance of this observation is unclear

Our studies using other inhibitors are less conclusive since the specificity of some of these agents is less well established (Karnovsky and Hug 63 Barron et al 63) particularly at the histochemical level Furthermore in some cases the degree of inhibition can be assessed only from quantitative studies which are often obtained under conditions that differ substantially from those employed in histochemical techniques The preceding factors which account for a large part of the difficulties encountered in the classification of esterases assume critical importance when the enzymatic activity is low and when more than one type of enzyme is present at the site where the final product of the histochemical reaction is localized

*Interstitial cells* A large number of interstitial cells rich in non specific esterase were observed throughout the rat heart Many of these cells were identified as pericytes which in other tissues have been found to contain large amounts of non specific esterase (Gomori 53) As in the muscle fibers two types of esterase activity cytoplasmic and lysosomal were demonstrated in the interstitial cells

The cytoplasmic esterase of the interstitial cells was better demonstrated by the α naphtholic than by the naphthol AS or indigogenic esters This enzyme reacted only very weakly with thiolacetic acid and did not react with any of the choline esters or with naphthol AS D chloroacetate It differed from the muscle fiber enzyme in several respects it reacted more intensely with naphthol AS esters it was less sensitive to the inhibitory effect of aldehyde fixation and following treatment with $10^{-5}$ M $E_{600}$ it was activated by PCMB to a much lesser degree than the cytoplasmic esterase of the muscle fibers However the two enzymes reacted in a similar manner to various inhibitors (table 2) These results indicate that as in the muscle fibers the major part of the non specific esterase activity of the cytoplasm is caused by a B esterase and that only a very sm[illegible] amount of C esterase is present in th[illegible] cytoplasm of the interstitial cells

The fluoride and $E_{600}$ resistant es[illegible] activity of the lysosomes present in th[illegible] cardiac muscle fibers and the intersti[illegible] cells could be shown with all the s[illegible] strates except the choline esters and [illegible] best demonstrated in sections in wh[illegible] the cytoplasmic esterase had been s[illegible] pressed by fluoride ions The biochemic[illegible] characteristics of this type of esterase a[illegible] tivity i e lack of inhibition by fluor[illegible] iodoacetate N ethylmaleimide and by r[illegible] atively high concentrations of organoph[illegible] phates are similar to those found by H[illegible] (63) in the lysosomes of other tiss[illegible] According to the evidences presented b[illegible] Hess and Pearse (58) Wachstein M[illegible] and Falcon (61) Sabatini Bensch a[illegible] Barrnett (63) and Holt (63) these ch[illegible] acteristics suggest that the hydrolysis [illegible] esterase substrates by lysosomes rath[illegible] than resulting from the action of es[illegible] ases may be due to cathepsins wh[illegible] are known to be present in lysosomes a[illegible] are capable of attacking a large variety [illegible] substrates Further studies will be nece[illegible] sary to investigate this possibility

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## PLATE 1

### EXPLANATION OF FIGURES

1 Cross section through the bundle of His 200 × Post fixation for 30 minutes in cold formol-calcium α naphthyl acetate Diazo Blue B Staining is most intense in the specialized fibers of the bundle and in the surrounding interstitial cells and nerve fibers

2 Left branch of the bundle of His 200 × Post fixation for 30 minutes in cold formol calcium α naphthyl acetate Diazo Blue B There is strong staining of the specialized muscle fibers and adjacent interstitial cells and nerve fibers

3 Atrioventricular node 200 × Post fixation for 30 minutes in cold formol calcium α naphthyl acetate Diazo Blue B Section through a portion of the atrioventricular node showing the staining pattern of the AV nodal fibers and their loose irregular arrangement

4 Bundle of His 600× Post fixation for 30 minutes in cold formol calcium α naphthyl acetate Diazo Blue B A moderately intense reaction is obtained in the fibers of the bundle of His


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## PLATE 2

### EXPLANATION OF FIGURES

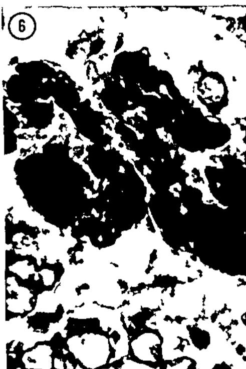
5 Right ventricular wall 1 000 × Post fixation for 30 minutes in cold Formol-calcium a naphthyl acetate Diazo Blue B Cross section showing moderately intense esterase activity in the cytoplasm of the ventricular muscle fibers

6 Right atrial wall 400 × Post fixation for 30 minutes in cold formol calcium a naphthyl acetate Diazo Blue B A strong esterase reaction is seen in the cardiac neurons their post ganglionic nerve fibers and in the cytoplasmic rim of adipose tissue cells

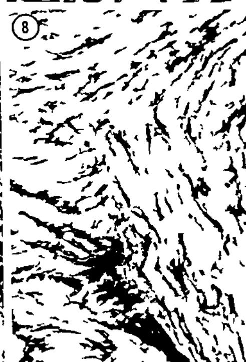
7 Base of the septal leaflet of the mitral valve 200 × Post fixation for 30 minutes in cold formol calcium a naphthyl acetate Diazo Blue B There is a large number of intensely stained interstitial cells in th connective tissue at the base of the valve

8 Upper part of the muscular portion of the interventricular septum 200 × Pre fixation for 24 hours in formol calcium a naphthyl butyrate hexazotized p rosanilin The staining and general distribution of the interstitial cells are observed The cytoplasm of the muscle cells shows no esterase activity


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## PLATE 3

### EXPLANATION OF FIGURES

9 Left ventricular myocardium 1 000 × Pre fixation for 24 hours in formol calcium a naphthyl butyrate Diazo Blue B There is very intense staining of the interstitial cells their cytoplasmic processes are less clearly evident than in figure 10 Very faint staining is observed in the muscle fibers

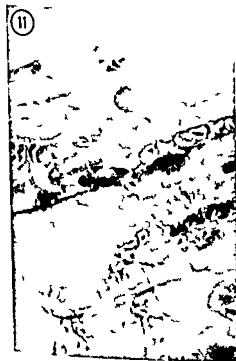
10 Left ventricular myocardium 1 000 × Pre fixation for two hours in cacodylate buffered glutaraldehyde a naphthyl acetate hexazotized p rosanilin Note the intensely stained pericyte and its cytoplasmic processes Distinct staining is also observed in the muscle cells

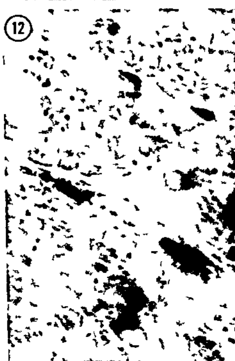
11 Left ventricular myocardium 1 000 × Pre fixation for two hours in cacodylate buffered glutaraldehyde a naphthyl acetate hexazotized p rosanilin Section preincubated with 0 01 M sodium fluoride The cytoplasmic staining of the muscle fibers and interstitial cells is completely inhibited The lysosomes of the interstitial cells remain intensely stained

12 Left ventricular myocardium 1 000 × Post fixation for 30 minutes in cold formol-calcium Naphthol AS D acetate Fast Garnet GBC salt 1 000 × The interstitial cells are strongly stained A discrete reaction is evident in the muscle fibers however the dye deposits are granular in character Compare with figure 10


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## PLATE 4

### EXPLANATION OF FIGURES

13 Epicardium and part of the wall of the left ventricle 600 × Pre fixation for 24 hours in formol-calcium α naphthyl butyrate hexazotized p rosanilin The subepicardial interstitial cells are intensely stained

14 Septal leaflet of the tricuspid valve 200 × Pre fixation for 24 hours in formol-calcium α naphthyl butyrate hexazotized p rosanilin A large number of esterase positive cells is present in the valve leaflet

15 Interventricular septum 200 × Pre fixation for 24 hours in formol calcium α naphthyl acetate hexazotized p rosanilin Numerous esterase positive cells are seen in the subendocardial connective tissue of the interventricular septum

16 Part of the media and adventitia of the aortic wall 600 × Pre fixation for two hours in cacodylate buffered glutaraldehyde α naphthyl acetate hexazotized p rosanilin There is a large number of intensely stained connective tissue cells in the adventitia of the aorta


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## PLATE 5

### EXPLANATION OF FIGURES

17 Left ventricular *myocardium 400 ×* Pre fixation for two hours in cacodylate buffered glutaraldehyde Holt s complete indigogenic medium employing 5 bromo-4-chloro-indoxyl acetate *There* is faint staining of the muscle fibers The interstitial cells show mainly lysosomal staining

18 Left ventricular myocardium 200 × Pre fixation for two hours in cacodylate buffered glutaraldehyde *Holt s indigogenic* medium employing 5 bromo-4 chlorindoxyl acetate but with the concen*tration of* the ferricyanide ferrocyanide redox system reduced from 0 005 to 0 0005 M There is a marked increase in the intensity of the staining of the muscle fibers *and of* the interstitial cells

19 Left ventricular myocardium *1 000 ×* Pre fixation for two hours in cacodylate buffered glutaraldehyde Holt s indigogenic medium employing 5 bromo-4-chloroindoxyl acetate and 0 0005 *M ferricyanide* ferrocyanide redox system Section pretreated with 0 01 M sodium fluoride *Staining* is now confined *to* the lysosomes of the interstitial cells

20 *Left* ventricular myocardium 200 × Pre fixation for 24 hours in formol-calcium Holt s indigogenic *medium* employing 5 bromo 4 chloroindoxyl acetate and 0 0005 M ferricyanide ferrocyanide *redox system* (as in figs 18 19) Prolonged fixation has caused a considerable decrease in the intensity of the esterase reaction of the muscle fibers

21 Left ventricular myocardium 200 × Pre fixation for 24 hours in formol calcium Holt s indigogenic medium *using* 5 bromo-4-chloroindoxyl acetate and 0 0005 M ferricyanide-ferrocyanide redox *system Section* pretreated with $10^{-5}$ M $E_{600}$ Comparison with figure 20 demonstrated the inhibition of the cytoplasmic staining of the muscle fibers and interstitial cells

22 Left ventricular myocardium 1 000 × Pre fixation for two hours in cacodylate buffered glutaraldehyde *Thiolacetic* acid medium Staining is mainly localized in the lysosomes of the subendocardial *interstitial* cells


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## PLATE 6

### EXPLANATION OF FIGURES

23 Section through the bundle of His and adjacent atrial and ventricular myocardium 100 × Post fixation for 30 minutes in cold formol calcium Holt s indigogenic medium using 5-bromo-4-chloroindoxyl acetate and 0 0005 M ferricyanide ferrocyanide redox system The most intense staining is observed in the elements of the conduction system staining of atrial muscle is greater than that of ventricular myocardium

24 Right atrial wall 100 × Post fixation for 30 minutes in cold formol calcium Holt s indigogenic medium using 5 bromo-4-chloroindoxyl acetate and 0 0005 M ferricyanide-ferrocyanide redox system The nerve cells and post ganglionic nerve fibers of a cardiac ganglion are intensely stained A moderate reaction is observed in the surrounding atrial muscle cells


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## PLATE 7

### EXPLANATION OF FIGURES

25 Bundle of His and surrounding areas 50 × Post fixation for 30 minutes in cold formol-calcium Myristoyl choline The bundle is intensely stained No staining is observed in the interventricular septum and only a very faint reaction is seen in the atrial muscle cells

26 Section serial to that shown in figure 25 50 × Post fixation for 30 minutes in cold formol-calcium Myristoyl choline after treatment with $10^{-5}$ M eserine Cholinesterase activity is completely inhibited by eserine

27 Same section as in figure 25 200 × Post fixation for 30 minutes in cold formol calcium Myristoyl choline There is intense cholinesterase activity in the right branch of the bundle

28 Bundle of His 400 × Post fixation for 30 minutes in cold formol-calcium Lauroyl choline The nerve fibers surrounding the bundle of His are intensely stained The specialized muscle cells show weak but distinct staining

29 Cardiac Ganglion 600 × Post fixation for 30 minutes in cold formol-calcium Acetylthiocholine iodide The nerve cells show intense cholinesterase activity

30 Cardiac Ganglion 600 × Post fixation for 30 minutes in cold formol-calcium Acetylthiocholine iodide Section pretreated with $10^{-5}$ M iso OMPA Comparison with figure 29 shows the reduction in cholinesterase activity produced by iso-OMPA


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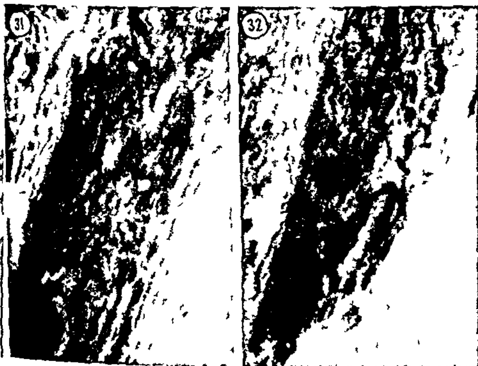

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## PLATE 8

### EXPLANATION OF FIGURES

31 Bundle of His and adjacent atrial and ventricular myocardium 200 × Post fixation for 30 minutes in cold formol-calcium Butyryl thiocholine iodide The most intense reaction is observed in the bundle of His (center) the atrial myocardium (upper left) shows more intense staining than the ventricular myocardium (lower right)

32 Section similar to that shown in figure 31 200 × Post fixation for 30 minutes in cold formol-calcium Acetylthiocholine iodide The staining pattern is the same at that obtained with butyrylthiocholine iodide



# ıstant Cell Populatıons ın Normal, Testosterone-
# ɔrıved and Testosterone stımulated
# ɾator Anı Muscles [1]


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ABSTRACT Castration of adult male mice produces a true atrophy of their levator ani muscles until the muscle reaches a stable weight within 45 days Subsequent treatment with testosterone produces a true hypertrophy from the stable neutered state The weight changes in the muscle are accounted for solely by changes in the size of the muscle fibers No mitotic activity accompanies atrophy or hypertrophy The number of cell nuclei and the amount of DNA in the muscle is unaltered


n the adult male mouse the mass of *levator ani* dwindles following castra ı then waxes after a parenteral ınjec ı of testosterone Concomitantly there marked changes in the diameters of muscle fibers (Wainman and Shipoun 41) Whether the change in muscle ss involves only the pre-existing mus fibers or includes new tissue compo- its has not been clearly established It generally accepted that mammalian letal muscles enlarge during the later ges of growth and following persistent rcise by a hypertrophy of the individual ıscle fibers the number of fibers remain ; constant (MacCallum 1898 Morpurgo 97) One wonders whether testosterone nsitive muscles accomplish their weight anges similarly Kochakian (64) has monstrated that enlargement of the stosterone-stimulated masseter muscle of e guinea pig involves no increase in NA implying no increase in the number nuclei Corollary information on the ntributions of the different tissue com ɔnents to the total muscle mass is lack ıg

Herein is evidence that the changing ıass in the *levator ani* is essentially a uctuation of sarcoplasmic mass in the ıuscle fibers This is demonstrated by our characteristics of the atrophied and ıypertrophied *levator ani* (1) the relation f the size of its muscle fibers to its total veight (2) the mitotic activity of its ells (3) the relative numbers of its dif erent cell types and (4) its total quantity of DNA

These characteristics are discussed in comparing the *levator ani* and other skel etal muscles during growth maintenance atrophy and hypertrophy The morpho- logical changes in the *levator ani* are de scribed in an accompanying report (Ven able 66)

## MATERIALS AND METHODS

Male white mice were obtained from the Charles River mouse farms North Wilmington Massachusetts They formed three experimental groups *intact neuter* and *testosterone treated neuter* groups The mice of the *intact* group were left uncastrated and received no testosterone The mice of the *neuter* group were cas trated as adults at 40 days of age and left untreated for 45 days to allow maximum atrophy of the *levator ani* (Venable 66) The mice of the *testosterone treated neuter* group were treated identically to the *neu ters* but subsequently received 0 5 mg of Depo testosterone (Upjohn) intramuscu larly All mice in any one experiment had the same date of birth experienced iden tical environmental influences and were sacrificed on the same day

For the comparison of muscle fiber size to total muscle weight mice from all three


[1] Research upported by N tion l Scien e Found tion F llow hip 2G-406 tr ining grant GM-406 and by gr nt GM 10182.


B ed n the i submitted to the Divi ion of Medi l Science The G du te School of Art and Sci nces H rv d University in p rti l f lfillment of th requ rements for th Degree of Doct r of Philosophy in An tomy


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groups — four *intact* six *neuter*, six *testosterone treated neuter* mice — were anesthetized with sodium pentobarbital killed by cervical dislocation, and their levator ani muscles dissected with blunt scissors under isotonic saline After blotting off the excess saline these muscles were weighed quickly and then fixed with glutaraldehyde (Gordon et al, 64) As with most aldehyde fixatives glutaraldehyde caused the muscle to swell Even when the swelling was dissipated in a normal saline bath the weight changes of the individual muscles were quite variable It proved important to use the weights of the unfixed muscles in correlations with muscle fiber size

To measure muscle fiber diameters fixed muscle samples were washed in normal saline and the individual fibers dissociated by low speed action of a micro blender Samples of dispersed fragments of muslcle fibers were stored in isotonic saline allowing osmotic equilibration before measurement on a standard light microscope equipped with a 25 × water immersion objective and a calibrated eyepiece micrometer The muscle fibers from the *levator ani* were of uniform diameter throughout the muscle therefore errors due to inappropriate sampling were avoided The diameters of 50 successively encountered fragments of muscle fibers were measured where they were most nearly cylindrical avoiding flattened regions Each diameter was halved to give a radius Each radius was squared and the arithmetic mean of the 50 squared radii ($\overline{r^2}$) served as the statistic to be compared with the weight of the muscle

The relationship between muscle fiber size and muscle weight can be formulated in the following manner The weight of the muscle (W) equals the weight of the muscle fibers (F) plus the weight of the interstitial tissues (C)

$$W = F + C \qquad (a)$$

The weight of the fibers (F) can be expressed as a function of the product of their mean number (N) their mean density (D) their mean length (L) and their mean cross sectional area ($\pi\overline{r^2}$) Substituting this approximation for F in equation (a) produces the equation

$$W = NDL\pi\overline{r^2} + C \qquad (b)$$

If N D L and C are constants equation (b) is a linear one with the useful characteristic of relating muscle weight (W) to the mean squared fiber radius ($\overline{r^2}$) Expressed in standard form this equation is of the type

$$W = m\overline{r^2} + b \qquad (c)$$

The degree of constancy of m and b can be estimated by a correlation coefficient (Snedecor 56) calculated from the data of muscle weight and mean squared fiber radii a high correlation coefficient indicating these terms to be relatively constant A high coefficient would then imply that changing fiber radius accounts for most if not all of the changing muscle mass

This equation was useful in this study because morphological observations (Venable 66) indicated that fiber length fiber density and connective tissue mass remained relatively constant The equation can be adapted to other situations to compare any two of its parameters or groups thereof provided that the remaining parameters can be estimated

Mitotic activity was sought both by microscopic examination of sections for mitotic figures and by administering tritiated thymidine to label nuclei synthesizing DNA The thymidine $H^3$ (6 05 c mM) was injected intraperitoneally at a dose of 1 0 μc per gram body weight A ... of radiographs of epon sections one and one-half microns thick were made by coating them with the Ilford L-4 nuclear emulsion (Hay and Revel 63) Development of the latent image with 1 part Dektol (Kodak) and 3 parts water followed a three week exposure of the emulsion

The relative number of nuclei of the different cell types was determined from epon sections three-quarter micron thick The accompanying report on the morphology of the *levator ani* (Venable 66) presents the technique of preparing the muscle for epon embedding and the criteria for identifying the cell types

For the determination of the total DNA in the muscle mice were decapitated the *levator ani* muscle dissected weighed minced and homogenized in a 1 ml homogenization tube The homogenate was washed subsequently in a 15 ml centrifuge tube with 1 ml of saline where the acid

:le fraction and phospholipid frac were extracted by the method of -eider (45) The remaining proteins ding the nucleoproteins contained be n 20 and 30 µg of DNA Colorimetric ysis of these quantities by the modified le method of Schmid et al (63) fol d Beer's Law closely This method d for hydrolysis of the nucleic acids 0 C for 80 minutes in 2 ml of 5% ochloroacetic acid adjusted to pH 2 45 NaOH The hydrolysis was stopped ing the reaction tubes One milliliter n 0 06% indole solution and 1 ml of HCl were added and the tubes in an 85 C water bath for an ad onal 80 minutes where colored products ndole pentose reactions formed These tions were stopped also by icing the tion tubes Spurious colored products orbing light in the range of indole-de ribose 490 mµ were removed by four actions with chloroform The remain optical density of the solution at 490 was measured with a Bausch and Lomb ctronic 20 spectrophotometer Control asurements included a reagent blank l duplicate samples of 30 µg of highly ymerized DNA from calf thymus (Sigma emical St Louis)

## RESULTS

*Relation between muscle fiber size and total muscle weight*

The degree of linear correlation between weight of the muscle and the mean uared radius of its fibers is high (cor ation coefficient equals 0 90) Figure presents a graph of the data An analysis variance attributes 55% of the total riation to the regression line The resid l variation of 45% includes variation in ssecting the muscles as well as varia ons in the assumed constants N (num r of muscle fibers) L (length of fibers) (density of fibers) and C (amount of nnective tissue) At the point on the gression line where the average squared dius of the muscle fibers equals zero a sidual muscle weight of 3 7 mg remains hich is a reasonable estimate of the eight of the connective tissues

The high degree of linear correlation etween fiber size and muscle weight indi ates that the product of N L and D approaches constancy There is the possibility that the product remains constant though each parameter varies but this is unlikely The majority of the fibers are known to run the full length of the muscle so L (mean length of fiber) is not altered appreciably Neither is it likely that D (density) makes any radical change because the morphology of the muscle fibers in the three groups are similar Therefore N must be a fairly stable number

*Lack of mitotic activity*

No mitoses are evident in approximately 20 000 sectioned nuclei One half of these nuclei are from animals that received colchicine intraperitoneally six hours prior to sarcifice Neither castration nor testosterone treatment produces mitotic division in any of the cell types in the muscle

Tritiated thymidine administered from the thirtieth through the forty second hour following testosterone treatment of three neuter mice labeled only one fibroblast among 1 000 cells In two neuter mice untreated with testosterone but receiving the tritiated thymidine the thymidine labeled one fibroblast and two endothelial cells the latter located in adjacent adipose tissue Though the time chosen for the administeration of the thymidine may have been inappropriate for demonstrating an early burst of mitotic activity in view of the other observations these negative results provide additional support for the concept of a static number of cells participating in the alterations in mass in the muscle

*Differential nuclear counts in primary muscle bundles*

Of 1 217 sectioned nuclei from two *intact* mice 57% belong to muscle cells 21% to endothelial cells 14% to fibroblasts 6% to pericytes and 2% to satellite cells Table 1 presents these percentages and the 95% *confidence intervals* corresponding to an assumption that the samples are from a binomially distributed set of like samples Infrequently encountered cells such as mast cells macrophages and Schwann cells have been ignored

Differential counts from two mice from the *neuter* group display a decreased fre

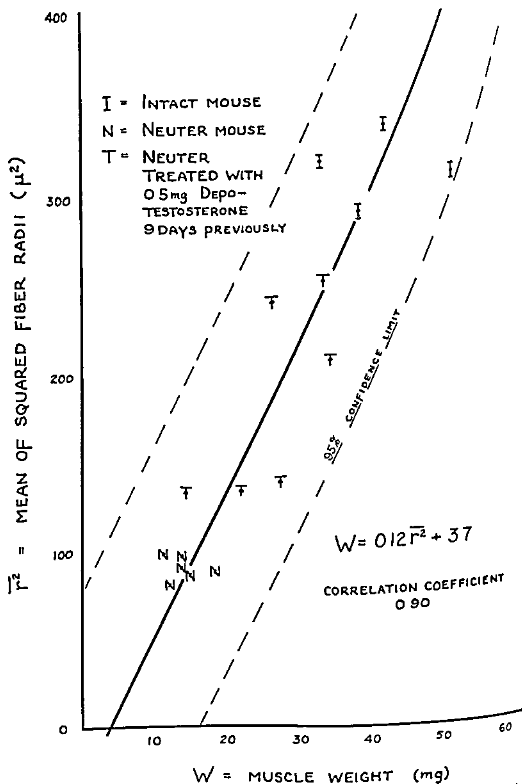



Fig 1 A comparison of muscle weights and means of squared muscle fiber radii in levator ani muscles from *intact neuter* and *testosterone treated neuter* mice The linear regression is plotted along with a 95% confidence limit calculated from the data The equation for the regression line is presented this being a unique characterization of the *levator ani* muscle of the adult mice studied It includes the factors of muscle fiber number density of fibers length of fibers and amount of stroma (see text) The calculated correlation coefficient is 0 90

TABLE 1

*[...]ency of encounter of the nuclear types in sections from two intact mice*[1]

| [...]ar type | Number | | 95% Confidence interval[2] |
|---|---|---|---|
| | | % | % |
| [...]le | 690 | 57 | 54–60 |
| [...]thelial | 257 | 21 | 19–24 |
| [...]blast | 165 | 14 | 12–16 |
| [...]yte | 78 | 6 | 5–8 |
| [...]llite cell | 27 | 2 | 1–3 |

[1] No correction is made for the three-quarter micron [...] of the sections or the dimensions of the [...] at right angles to the plane of the section [...]crombie 46). The percentages are rounded off [...] nearest whole number.
[2] The confidence intervals are selected from [...]licor's (56) table 1.3.1.

[...]ncy of encounter of endothelial cell [...]lei (16%) and a corresponding in[...]ase in the frequency of encounter of [...]scle nuclei (62%). The differential [...]nts from *testosterone treated neuters* [...]wever are remarkably similar to those [...] the *intact* group. The null hypotheses [...]t there are no differences in the differ[...]tial counts of the *nueter* and the *testos[...]one treated neuter* as compared of the [...]nt in the *intact* mice has been tested [...] the chi-square method. The data and [...] chi-square calculations appear in table [...]

The *neuter* is significantly different from [...] *intact* mouse in the frequency of en[...]unter of muscle nuclei ($P < 0.005$ one degree of freedom and of endothelial nuclei ($P < 0.005$ one degree of freedom). This might be interpreted as a change in cellular populations but this seems doubtful considering the insignificant difference between the counts from *testosterone treated neuter* and *intact* mice. It is much more likely that in the neuter state the subtle geometric changes in nuclear orientation and perhaps nuclear size is sufficient to change the probability of the inclusion of these nuclei in sections three quarter micron thick (Abercrombie 46).

### *Constant DNA content of the levator ani*

Considering the lack of mitotic activity and the similar proportions of the several cell types in the *intact* and *treated neuter* mice it was predicted that the total DNA content of the muscle would be independent of both castration and testosterone treatment.

To test this prediction the *levator ani* muscles from five *intact* five *neuter* and five *testosterone treated* mice were analyzed for their total content of DNA. The *treated neuter* mice had received their parenteral testosterone nine days previously. Table 3 presents the data.

The weights of the muscles fall into three distinct groups: the heavy muscles of the *intact* mice (45.3 mg), the light

TABLE 2

*Frequency of encounter of the nuclear types in the three experimental groups and calculation of chi squares*[1]

| Group | Nuclei: Muscle | Endothelium | Fibroblast | Pericyte | Satellite cell | Total |
|---|---|---|---|---|---|---|
| Intact | | | | | | |
| Number | 690 | 257 | 165 | 78 | 27 | 1217 |
| Per cent | 56.7 | 21.1 | 13.6 | 6.4 | 2.2 | |
| Neuter[2] | | | | | | |
| Number | 1093 | 290 | 211 | 128 | 46 | 1768 |
| Per cent | 61.8 | 16.4 | 12.0 | 7.2 | 2.6 | |
| Treated neuter[3] | | | | | | |
| Number | 450 | 160 | 117 | 55 | 21 | 809 |
| Per cent | 56.4 | 19.7 | 14.5 | 6.8 | 2.6 | |

[1] $\chi^2 = (f - F)^2/F$ where f equals the hypothetical number of nuclei calculated from the per cent ge found in the intact mice and F equals the observed values.

[2] $\chi^2 = \frac{(1093 - 1002)^2}{1002} + \frac{(290 - 373)^2}{373} + \frac{(211 - 241)^2}{241} + \frac{(128 - 113)^2}{113} + \frac{(46 - 39)}{39}$

$\chi = 8.264 + 18.469 + 3.734 + 1.991 + 1.256 = 33.714$ null hypothesis rejected $P < 0.005$

[3] $\chi = 0.020 + 0.588 + 0.445 + 0.173 + 0.500 = 1.726$ null hypothesis accepted $P < 0.005$

TABLE 3

*Total DNA in the levator ani muscles of intact neuter and testosterone treated*[1] *neuter mice*

| Animal | Group | Body weight | Muscle weight | Total DNA | Means and standard deviations: Body weight | Muscle weight | Total DNA |
|---|---|---|---|---|---|---|---|
| | | *gm* | *mg* | *µg* | *gm* | *mg* | *µg* |
| 1 | Intact | 46 0 | 51 5 | 24 9 | | | |
| 2 | | 44 0 | 46 6 | 25 0 | | | |
| 3 | | 44 5 | 42 4 | 21 0 | | | |
| 4 | | 44 0 | 38 7 | 20 5 | | | |
| 5 | | 45 5 | 47 5 | 28 2 | 44 8±0 9 | 45 3±4 9 | 33 3 |
| 6 | Neuter | 43 5 | 16 4 | 23 6 | | | |
| 7 | | 35 0 | 12 9 | 20 3 | | | |
| 8 | | 36 0 | 16 0 | 27 5 | | | |
| 9 | | 41 0 | 14 6 | 15 5 | | | |
| 10 | | 35 0 | 16 7 | 28 3 | 38 1±3 9 | 15 3±1 6 | 23 0 |
| 11 | Neuter treated with testosterone | 46 0 | 31 4 | 28 6 | | | |
| 12 | | 39 0 | 31 2 | 25 9 | | | |
| 13 | | 43 0 | 24 8 | 21 5 | | | |
| 14 | | 50 0 | 36 7 | 24 5 | | | |
| 15 | | 39 0 | 28 2 | 24 3 | 43 4±4 7 | 30 5±4 4 | 48 |

[1] 0 5 mg Depotestosterone (Upjohn) nine days previous to sacrifice

muscles of the *neuters* (15 3 mg) and the intermediate muscles of the *neuters treated with testosterone* (30 5 mg) The total DNA content of the muscles varies between 20 and 29 µg except for one unusually low reading of 15 5 µg But there is no significant difference between the means representing the three groups This does not mean that the number of cells in this muscle is stable throughout adulthood Indeed, when data from adult mice of widely varying ages are compared differences are noted But it is clearly evident that the cell number is independent of the effects of castration and testosterone treatment

DISCUSSION

The concept of a stable number of skeletal muscle fibers forming early in mammalian life is well supported MacCallum (1898) has reported that the fixed number is established before birth in the human *sartorius* This is questioned by Montgomery ( 62) who finds new fibers forming postnatally In the mouse Goldspinck ( 62) also notes formation of new muscle fibers postnatally Taken as a whole the evidence presented indicates a stabilization of fiber number relatively early in the growth period This constant number of fibers persists into aldulthood and is refractory to change even during the muscular hypertrophy of exer[illegible] (Morpurgo 1898) The data from the [illegible] *levator ani* confirms this constancy [illegible] ipulation of testosterone levels can not [illegible] ter the established number of muscle [illegible] bers

Though the number of fibers in an a[illegible] remain unchanged there is growing [illegible] dence that the number of muscle n[illegible] continually increase Mongomery ( [illegible] reports a nuclear increase in the hu[illegible] *sartorius* from birth through the se[illegible] decade of life He hypothesizes a c[illegible] tinual fusing of an expanding popul[illegible] of myoblasts into existing muscle fi[illegible] The studies of skeletal muscle made [illegible] Morpurgo (1898) Morpurgo and B[illegible] (1898) and Schiefferdecher ( 09) provi[illegible] many instances of an increasing numbe[illegible] of muscle nuclei as muscles age Th[illegible] postulate amitosis of muscle nuclei as th[illegible] cause Enesco and Puddy ( 64) co[illegible] of these observations present a his[illegible] metric study of skeletal muscles in the [illegible] that demonstrates constancy in the num[illegible] ber of muscle fibers and yet a 10 to [illegible] fold increase in the number of musc[illegible] nuclei from the sixteenth to the e[illegible] fourth day of age MacConnachie En[illegible] and Leblond ( 64) working with the sa[illegible] material demonstrate that these nuclei [illegible] corporate thymidine and divide mitoti[illegible] at rates in accordance with their increas[illegible]

numbers The nature of these mitotically active cells is not fully established MacConnachie notes that in many they resemble the "satellite cells of le fibers first described by Mauro ) and now recognized to be a con nt cell type in many striated muscles od 64 Midsukami 64 Muir et al Venable 64 66) These observa tempt speculation that the slowly in sing number of nuclei in muscle fibers esents the latent proclivity to myo sis that can be demonstrated by in ng mature skeletal muscles (Allbrook Walker 63 Zhinkin and Andreeva Price et al 64) and that this proclivity may burst forth when the *levator* is stimulated hormonally to produce exceptionally large number of muscle lei This speculation proves untenable ugh the number of muscle nuclei in *levator ani* is increasing slowly as in er mature skeletal muscles (limited published data on DNA content) the of increase is altered negligibly by tration and subsequent testosterone atment Mice in the three experimental ups analyzed show equal quantities of IA in their muscles because they were rificed at identical ages

Kochakian (64) in his study of the nu ic acid content of the *masseter* and *nporalis* muscles of growing guinea pigs tes that testosterone "retards the attain nt of the adult level of DNA" but does ot prevent it The implication is that the *asseter* and *temporalis* either assume a nstant quantity of DNA or the accumu tion slows to an immeasurable rate his adult level" is unaltered by adminis ring testosterone

The concept of Stockdale and Holtzer 61) that synthesis of DNA and synthe s of specific muscle proteins are antag nistic processes" is supported by the sponse of the mature *levator ani* to testos rone In this instance the muscle fibers re differentiated to synthesize maintain nd perhaps destroy sarcoplasmic proteins During atrophy these muscle cells o not dedifferentiate (Venable 66) and herefore their nuclei are mitotically dor nant as if locked to processes associated with functional muscle cells During eenlargement the increase in muscle mass is wholly the response of these same fully differentiated muscle cells so here also mitotic activity is repressed

It may seem remarkable that the endothelial cells of the capillary beds and the fibroblasts of the connective tissues are unresponsive to the considerable change in girth of the muscle fibers This be comes less surprising if one realizes that the capillaries and the connective tissues are more sensitive to changes in the lengths of muscle fibers than to changes in their girths This is so because capillar ies and connective tissue elements are ori ented primarily in parallel with the longi tudinal axis of muscle fibers In the atrophying and hypertrophying *levator ani* the length of the muscle changes very little As the girths of the muscle fibers decrease capillaries fibroblasts and col lagenous fibers are brought more closely together As the girths increase these ele ments move apart

The distances metabolites must diffuse from capillaries to deeply situated meta bolic sites in the sarcoplasm does not seem to limit total muscle mass provided the muscle fibers remain smaller than 70 μ in diameter the largest measured in this study

The conclusions drawn are these Testos terone alters neither the total number of nuclei nor the total number of muscle cells in the *levator ani* of mature mice though there is evidence from studies in guinea pigs that the rate of the accumulation of nuclei during the growth period may be affected This androgen s stimulatory ef fect is associated specifically with the syn thesis and maintenance of the sarcoplas mic mass of the muscle fibers (see Ven able 66)

Castration of a mature male mouse produces true atrophy of the muscle fibers of the *levator ani* Subsequent administra tion to the stable neuter produces true muscular hypertrophy

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# Morphology of the Cells of Normal, Testosterone Deprived and Testosterone stimulated Levator Ani Muscles [1]

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ABSTRACT The atrophy of the *levator ani* following castration of an adult mouse or rat results from the dissolution of the myofilaments from myofibrils and eventual loss of two-thirds of the sarcoplasm Within 45 days the atrophy ceases The morphology of the now smaller muscle fibers is indistinguishable from those from intact males Treatment of the stable neuter with testosterone produces rapid enlargement of the muscle fibers but negligible changes in the morphological patterns of th sarcoplasm. Observations indicate that new myofilaments form in register along the myofibrils the enlarging myofibrils being divided to maintain a diameter between 0 5 and 1 5 μ The cytology of each of the five cell types common to the primary bundles is described. Satellite cells of the muscle fibers are a consistent component, but do not contribute to the processes of atrophy or hypertrophy

In 1935 Papanicolaou and Falk reported administering testosterone to female and neuter male guinea pigs to stimulate enlargement of their masticatory muscles to a size characteristic of the intact male Three years later Wainman and Shipounoff (41) reduced the weight of the perineal muscles in male rats by castration This wasting could be prevented by administering testosterone Testosterone treatment of intact males resulted in abnormal enlargement of their perineal muscles The individual muscle fibers seemed to change their size harmoniously with the change in size of the muscle as a whole Surprisingly there was no alteration in the internal structure of the muscle fibers resolvable by light microscopy No mitotic activity was noted but the number of nuclei per unit area of section seemed to be greater in samples from the wasted muscles This was ascribed either to a crowding to either of the original nuclei or possibly to an infiltration of connective tissue cells

In 1949 Eisenberg et al treated the neutered male rat with testosterone and measured the remarkable increase in weight of a single muscle of the perineal complex the *levator ani* They suggested this measurement as a biological indicator of myotropic activity of androgens Subsequently Kochakian (59) reported a similarly induced weight increase in the *levator ani* of the mouse Other organs of the mouse such as the lacrimal and salivary glands urinary bladder and kidney also enlarged discernibly under the influence of parenterally administered testosterone

Because fine structural changes in muscles responsive to testosterone remained unknown and these changes seemed essential to understanding maintenance and growth of muscle tissue a re-examination of the morphology of the atrophying and enlarging *levator ani* was undertaken utilizing both light and electron microscopy of thin sections of the muscle embedded in epoxy resin This report presents the microscopical observations made during the study A preceding report (Venable 66) demonstrates the stability of the cell populations involved in these phenomenon and shows the changing weight of the muscle to be due solely to a change in sacroplasmic mass

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muscle is embedded in dense deep ineal and coccygeal fascia and is con cted posteriorly with the *external anal* hincter Figure 5 demonstrates these ationships

Histologically the *levator ani* is a typical ite skeletal muscle (figs 6 12) Its rallel cross striated muscle fibers range diameter from 10–70 μ in both adult ale mice and rats The frequency dis bution of fiber diameters is skewed ghtly positively with a mean and stand d error of the mean of 36 1 ± 0 5 μ (fig 1) Single fibers have a fairly uniform ameter throughout their length

Serially and sagittally cut celloidin sec ons of one half the *levator ani* from one ale mouse demonstrate that most mus e fibers extend the full length of the ecimen with the exception of the fibers sitioned at the outer margins of the uscle These latter ones end short of the rethral insertion and may not reach the id-line Whether or not fibers cross the udline dorsal to the rectum was not in estigated directly but the lack of a visi le raphe coupled with a lack of fibers nding in the near mid sagittal sections uggests that they are continuous

The muscle is primarily closely packed uscle fibers The meager collagenous onnective tissue recognizable by light mi roscopy is confined to a perimysium form ing the adventitial sheaths of the distribu he blood vessels and nerves Elastic fibers re confined to the walls of arteries The apillaries are plentiful and regularly spaced Most run parallel to the long axis f the muscle but are interconnected at ccasional intervals by oblique anasto-moses In cross sections of the muscle ach muscle fiber abuts at least two and usually three capillaries

Other than the muscle fibers the four common cell types found in every primary muscle bundle are in order of their fre quency of occurrence the endothelial cell of the capillary the fibroblast the pericyte (undifferentiated perivascular cell) and a cell appearing as a satellite to the muscle fi bers The latter seemingly identical to the cell described by Mauro (61) will be referred to as "the satellite cell" Histio-cytes and mast cells are sometimes en countered but are present more typically in the perimyseal connective tissues Mye linated nerves and Schwann cells are seen occasionally particularly since the chosen region for sampling often includes motor end plates The quantitative distribution of 1170 nuclei of cells in the primary bundles of muscles of three *intact* mice is presented in figure 1 In the accompany ing report (Venable 66) data is presented showing a constancy of the nuclear types in all of the three experimental groups of mice The percentages of encounter seen in the three intact mice are muscle nu clei (57%) endothelial nuclei (20%) fibroblast nuclei (14%) pericyte nuclei (7%) and satellite cell nuclei (2%) These data are not modified to account for differences in average nuclear size and sec tion thickness (Abercrombie 46)

*The morphology of the muscle fiber*

In electron micrographs the bulk of the muscle fiber is a continuum of myofila ments divided into anastomosing bundles by many thin longitudinally oriented planes of the remaining sarcoplasm (fig 12) These bundles the myofibrils present poly gonal and often asymmetrical cross-sec tions Their widths range from 0 5–1 5 μ in both the mouse and rat These myofi brils exhibit the banding and sarcomere structure recognized as typical of verte brate skeletal muscle (Huxley 57)

Sarcoplasm between the myofibrils is usually scant It exhibits mitochondria a reticulum of membranous tubes — the sarcoplasmic reticulum — and many small granules of varying size (figs 12 13)

Most mitochondria are scattered through out the sarcoplasm appearing singularly or in rows along myofibrillar interspaces The majority of sarcomeres make no direct contact with mitochondria A few mito-chondria aggregate either beneath the sar colemma or at the poles of the muscle nuclei These small subsarcolemmal aggre gates generally represent sites close to capillaries Mitochondria are more numer ous in the muscles from mice than in the muscles from rats an expected observation in view of Gauthier and Padykula s cor relation (63) between increased concen tration of mitochondria and decreased body mass

## MATERIALS AND METHODS

To avoid the superposition of the effects of normal muscle growth on the effects of testosterone the levator ani muscles of only adult male mice and rats were studied All the mice (55) were young adults The rats were either young adults (12) or old neuters (10)

Both the young adult mice and the young adult rats were divided into three groups the *intact neuter*, and *testosterone treated neuter* groups *Intact* animals were uncastrated and received no testosterone *Neuter* animals were castrated as adults at 40 days of age and maintained under standard animal room conditions long enough to allow near maximal atrophy of the *levator ani Testosterone treated neuter* animals were likewise castrated as adults and maintained for a minimum of 40 days, but this group then received an intermuscular injection of Depo testosterone (Upjohn) At the time of sacrifice all young adult mice were between 80 and 100 days old all young adult rats between 95 and 150 days of age

Two *young adult neuter* rats were sacrificed to study the fine structure of atrophying muscle fibers one at 22 days and one at 45 days following castration

The ten *old neuter rats* had been castrated prior to puberty and were approximately 300 days old Five were treated with Depo testosterone the others served as untreated controls

Preparation of the *levator ani* for morphological study included exposing it by blunt dissection through the excised perineum and fixing it *in situ* by flooding the dissected space with a 0 5–4 0% solution of glutaraldehyde buffered to pH 7 4 by 0 05–0 2 M sodium cacodylate Mosquito forceps attached to the rim of the anus and suspended by tape from a position above and behind the dorsally recumbant animal produced a slight tension on the muscle during fixation After 30 minutes exposure *in situ* to the fixative the muscle was dissected free and placed in fresh cold fixative Samples were taken from the fixed muscle at a site located originally just lateral to the rectum (fig 5) These were left in the fixative for an additional three hours

The samples were washed subsequently in several changes of a cacodylate-sucrose buffer solution (Gordon Mille and Bensch 63) in which they were left over night and then treated 1 to 2 hours the following morning with a 1% solution of osmium tetroxide in fresh washing solution Rapid dehydration through increasing concentrations of cold ethanol and embedding in Epon 812 as described by Luf (61) completed the preparation of the samples for sectioning

In a few instances muscles were fixed *in situ* with 1% osmium tetroxide buffered with 0 1 M phosphate or s-collidine instead of the glutaraldehyde osmium fixation

For light microscopy sections were cu which averaged one square micron in area and one half to three quarters of a micron in thickness These were stained with 1% toluidine blue in 1% borax and the entire section imaged on a 4 × 5 photographic film using a 25 × plan apochromatic (Zeiss) microscope objective Enlarged prints of these developed films served as work sheets Types of nuclei identified on the original section by means of a 100 × Neofluor Phase Objective (Zeiss) wer recorded on the large photographic print and provided the data on frequency o occurrence of nuclear types

Silver to gold thin sections for electron microscopy were stained with lead cacody late (Karnovsky 61) or lead citrate (Ven able Coggeshall 65) — with and withou previous treatment with uranyl acetate – and photographed in a RCA EMU 3E or 3 electron microscope

Measurements of muscle fiber diameter were made from glutaraldehyde fixed sar ples taken from regions near the sectione samples and from similar areas from th opposite half of the muscle The techniqu is described in the preceding article

## OBSERVATIONS

The *levator ani* in the male mouse ar rat is a loop of muscle forming a coll dorsally and laterally around the last fe millimeters of the rectum Ventral to th rectum the two ends of the loop attach two paired caudal extensions of the fibrou capsule of the urethral bulb The musc lar loop and the fibrous capsule complete encircle the rectum Dorsal to the rectu

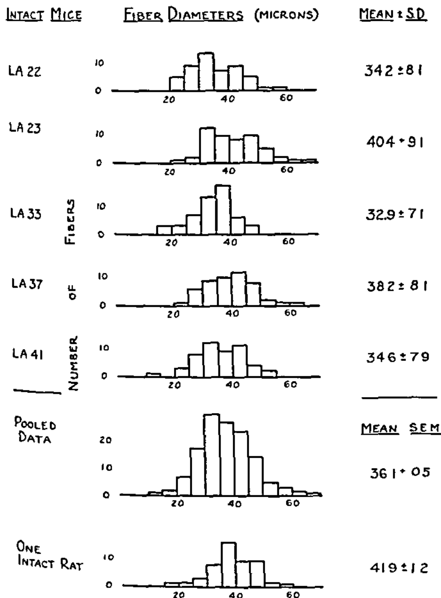



Fig 2 Histograms of muscle fiber diameters of the *levator ani* of five *intact adult mice* and one *intact adult rat* The much larger size of the *levator ani* muscle in the rat is due to a larger number of muscle fibers rather than larger fibers

bands A very small number of triads run longitudinally across A bands In *old neuter rats* it is common to find occasional pentads (Revel 62) These are not found in the muscles of younger animals

Granules are numerous in the sarcoplasm They average 25 mμ in diameter with a range of 10–40 mμ They have an affinity for lead stains Uranyl acetate staining alone adds little contrast to these granules — or to any other structures in these glutaraldehyde-osmium fixed muscle fibers A very few multilocular granules can be found One might assume all the

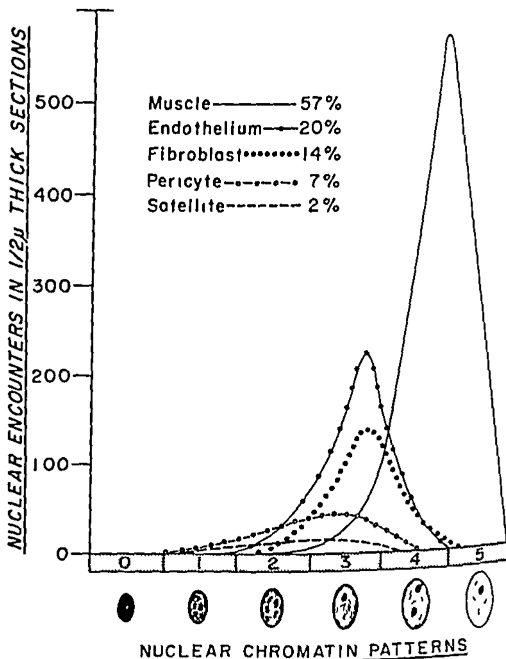



Fig 1 Relative numbers of nuclei of the five cell types common to primary muscle bundles of the *levator ani* of three adult male mice Variations in the chromatin patterns common to the nuclear types is indicated by position on the abscissa Examples of the chromatin patterns are found in figure 8 through 11 where they are labeled by the number system appearing here

The sarcoplasmic reticulum of the *leva tor ani* is morphologically similar in basic respects to that described by Porter and Palade ( 57) for the rat and now regarded as typical for white fibers of mammalian skeletal muscle A longitudinal system of tubules closely invests each myofibril and is fused to them at the Z-lines The transverse system of tubes or T system (... son Cedergren 59) is apparent in association at the A–I junctions with transverse cistern formed by ... of the more longitudinally oriented sar... plasmic tubules The transversely orga... ized triads appear at 75% of the bord... of the longitudinally sectioned A and

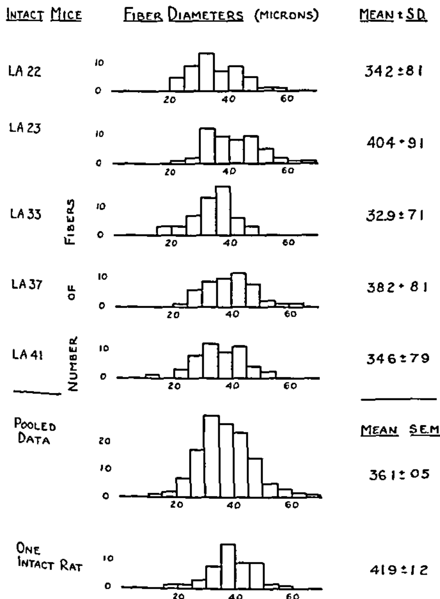



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granules to be glycogen on the basis of these characteristics (Revel 64) but due to uncertainties as to the effect of the glutaraldehyde fixation on these staining characteristics the possibility that some or many may be ribosomes can not be excluded

*Muscle nuclei* are typically located at the periphery of the muscle fibers though a few are buried among the myofibrils They are shaped most often like elongated platters Their average length is 16 μ their average width measured tangentially and transversely to the fiber is 7 μ their average depth is 2 5 μ The glutaraldehyde osmium fixed muscle nuclei show a thin rim of densely stained chromatin at the periphery of the karyotheca but only a few chromatin strands scattered in the karyoplasm The thin rim of chromatin is more electron opaque than that seen in muscle nuclei fixed by osmium alone though the patterns of chromatin clumping seem identical One to three per cent of the muscle nuclei have thicker, coarser patterns of chromatin than the rest Such patterns are similar to those characteristic of satellite cell and pericyte nuclei

Muscle nuclei invariably are within 30 mμ of the myofibrils in this mature muscle a space unresolved by light microscopy This is a useful criterion for differentiating muscle nuclei from nuclei of indenting satellite cells fibroblasts and endothelial cells

*The satellite cell*

Satellite cells must be considered normal components of the *levator ani* These cells lie inside the muscle fiber s glycocalyx (of Bennett 63) often referred to as the basement membrane yet are distinctly outside the sarcolemma (plasmalemma) They nestle into the muscle fiber so as to leave the fiber s outer contour relatively undistorted (fig 7) The plasmalemmae of the satellite cell and the muscle fiber lie within 30 mμ of each other with no intervening glycocalyx

The cytoplasm of the satellite cell in the mature *levator ani* is usually sparse and shows little specialization It contains a small but distinct Golgi complex a few scattered smooth vesicles and tubules a pair of centrioles a few scattered mitochondria, and cytoplasmic granules suggestive of both glycogen and ribosomes (fig 14) The cytoplasm contains no granular endoplasmic reticulum

A striking differential characteristic of the satellite nucleus more readily discerned after glutaraldehyde-osmium fixation than osmium fixation alone is the coarse pattern of chromatin clumping The chromatin is in thicker strands and clumps in the karyoplasm and is in thicker aggregates at the karyotheca than that characteristic of the usual muscle cell nucleus

With thinly sectioned glutaraldehyde osmium fixed specimens it is possible to differentiate between satellite cells and muscle nuclei by light microscopy (figs 7 10) Stained sections one half to three quarters of a micron in thickness are scanned at a magnification of 400 Nuclei lying within the outer contours of the muscle fibers which exhibit coarser chromatin patterns than those of usual muscle nuclei are critically examined for a narrow light line less than 0 4 μ in thickness between the karyotheca and the dark stained A bands of the adjacent myofibrils Such a light line can not be resolved between muscle nuclei and adjacent A bands Encroaching fibroblasts and endothelial cells have a wider space between their nuclei and the nearest myofibrils of the muscle cell It is usually possible to see the cytoplasm of the satellite cell contrasted against the sarcoplasm since the former s staining and optical properties differ somewhat from the latter Using these criteria approximately 4% of what otherwise could be mistaken easily for muscle nuclei are more accurately identified as nuclei of satellite cells

The satellite cell nuclei average 13 μ in length 4 5 μ in width and 2 8 μ in depth They are thus smaller and more fusiform than muscle nuclei By being smaller they have a lower probability of appearing in sections (Abercrombie 46) making the figure of 4% satellite nuclei given above an underestimate Adjusting for the decreased probability of their appearance in sections the true percentage of satellite cell nuclei to muscle nuclei in the *levator*

of adult mice likely falls between 4 8 1 5 8%

*The pericyte*

Pericytes (Rouget 1873) are very similar morphologically to satellite cells (fig 16) They do differ however in three characteristics their position in relation to the muscle cells their slightly coarser pattern of chromatin and their completely circumscribing glycocalyx

The pericyte s differentiation by light microscopy depends upon its adjacency to capillaries its paucity of cytoplasm and coarseness of chromatin pattern On electron micrographs the pericyte has the additional identifying characteristic of a glycocalyx This glycocalyx is unattached to the one surrounding the adjacent capillary Cytoplasmic fine structure of the pericyte is identical to that of the satellite cell

The pericytes of the capillaries and the satellite cells of muscle fibers are indeed so similar in morphology that it is feasible to consider them to be two forms of the same cell. This feasibility is strengthened by instances of pericytes indenting muscle fibers where the two usually separate glycocalyces are fused to form a single line In figure 14 the glycocalyx dips between the satellite cell and muscle fiber existing first as a double line then as a single fused membrane and finally disappearing leaving the plasma lemmae in close apposition Figure 15 shows a longitudinal view of a nested satellite cell or perhaps a pericyte Here the glycocalyces are double in some areas and single fused membranes in others Early in this study it was speculated that perictyes become satellite cells during the testosterone stimulated hypertrophy of the muscle However the quantitative data indicate no significant shift between these two cell populations

*The fibroblast*

By light microscopy fibroblasts sometimes prove difficult to differentiate from pericytes This is particularly true on cross sections of the muscle where the usual longitudinal streaming of the cytoplasm from both ends of the fibroblast nucleus does not appear in the section In these instances one must rely for its differentiation on the lighter staining surrounding cytoplasm and a usually less dense chromatin pattern In electron micrographs by contrast fibroblasts are identified easily for they are devoid of a glycocalyx (fig 16) and present a notable amount of a granular endoplasmic reticulum

*The endothelial cell*

Endothelial cells form capillaries typical of those in other muscles (Fawcett 63) Their nuclei are easily identified by light microscopy in sections one half to three quarters of a micron thick for the boundries of capillaries can always be distinguished with certainty (figs 6–11) This is seldom the case when the muscle is studied in thicker paraffin sections

*The levator ani of the neuter*

Atrophy of the *levator ani* following castration of the male mouse and rat involves a loss of myofilaments from muscle fibers but no frank degeneration of muscle tissue Table 1 compares the muscle weights of *intact mice neuter mice cas*

TABLE 1

*Atrophy of the levator ani of the mouse 45 days and six months after castration* [1]

| | Intact control | | 45 Day neuter | | Six month neuters | |
|---|---|---|---|---|---|---|
| | Muscle weight | Body weight | Muscle weight | Body weight | Muscle weight | Body weight |
| | *mg* | *gm* | *mg* | *gm* | *mg* | *gm* |
| | 51 5 | (46 0) | 16 4 | (43 5) | 14 2 | (66 5) |
| | 46 6 | (44 0) | 12 9 | (35 0) | 16 0 | (54 0) |
| | 42 4 | (44 5) | 16 0 | (36 0) | 15 7 | (41 0) |
| | 38 7 | (44 0) | 14 6 | (41 0) | 12.5 | (44 0) |
| | 47 5 | (45 5) | 16 7 | (35 0) | 13 0 | (56 0) |
| Mean muscle weight ± S D | 45.3 ± 4 9 | | 15.3 ± 1 6 | | 14.3 ± 1 6 | |

[1] The f to t nimals nd 45-d y neuter w re 99 d y old The six month neuters were 227 days old. Both groups f n uters were ca tr ted when they were 43 d y old.

trated for 45 days and *neuter mice castrated for six months* By the forty fifth day after castration the muscles from *neuter* males have reached a new stable weight one third that found in *intact* males Little, if any further atrophy occurs

A comparison between electron micrographs of levator ani muscles of *neuter* males castrated for 45 days and micrographs from muscles of *intact* males shows no remarkable difference in morphology other than a decrease in the average diameter of the muscle fibers In the castrated mouse the average diameter drops from 36 1μ to 20 8 μ (fig 3)

A few samples were collected and examined during the earlier stages of atrophy in one rat a young adult castrated for 22 days These samples exhibit alterations from the *intact* and *neuter* morphology that seem to characterize the process of atrophy These alterations are a fraying of myofilaments from myofibrils a disorganization of the sarcoplasmic reticulum and a wrinkling of the sarcolemma and nuclear envelope Thick myofilaments disperse first from their interconnections at the M line (fig 17) then appear free in the sarcoplasm Additional sarcoplasmic space appears between fraying myofibrils The sarcoplasmic reticulum in these areas is often either absent or has lost its intimate association with the myofibrils These alterations are not found uniformly throughout the muscle some fibers appear to be normal The myofibrils in the muscle fibers of *neuters* tend to be of smaller average diameter than those in muscle fibers of *intact* animals

*The levator ani of the testosterone treated neuter*

Fifteen days following treatment of two *old neuter rats* with 1 mg of Depo-testosterone (2 μg per gram body weight) their levator ani muscles had increased in weight 2 3 times those of two untreated controls (table 2)

The same dose in young adult mice is much less effective A dose of 12 μg per gram body weight however produces within nine days an enlargement twice that of untreated controls (Venable 66) Response to the treatment seems to be

TABLE 2

*Muscle weights and muscle fiber diameters in old neuter rats treated with Depo-testosterone*[1]

| Animal | Group | Muscle weight | Fiber diameter (Mean ± S.E.M.) |
|---|---|---|---|
| | | *mg* | μ |
| LA 17 | untreated | 71 0 | 19 3±11 |
| LA 19 | untreated | 74 6 | 21 0±15 |
| LA 18 | treated | 174 9 | 27 6±13 |
| LA 20 | treated | 156 4 | 3[illegible] 9±18 |

[1] Two micrograms per gram body weight 15 days previous to sacrifice

varied as might be expected from variable rates of absorption of the repository drug used however the hypertrophy is in every case remarkable

Figure 4 compares the muscle fiber diameters of the *treated* and *untreated neuter mice* The histogram of fiber diameters from the treated muscles is lower and wider than that of the untreated ones — the mean and maximum fiber diameter of the treated muscles has shifted to the right some 40% above that of the untreated ones This new position is intermediate between that of *neuter* and *intact* animals

Electron micrographs of treated muscles show no striking structural alterations associated with this rapid enlargement There is a seeming increase in the average myofibril diameter but it is too small to explain the greatly expanded girth of the muscle fibers There is no great number of very small myofibrils to indicate new myofibrils forming by aggreation of new myofilaments Neither is there randomly scattered single myofilaments A site for the formation of myofilaments can not be detected

The presence of many incomplete intermyofibrillar septa of sarcoplasm (fig 12) suggests that existing myofibrils are being split In some instances septa seem to be invading single I bands Micrographs can give no direct evidence of a dynamic process such as myofibrillar splitting but as will be explained in the Discussion this and other related evidence points to a conclusion that the myofibrillar mass is continuously subdivided as it expands

Components of the sarcoplasm i e mitochondria sarcoplasmic reticulum and sarcoplasmic granules do not alter their structure descernibly during muscle en-

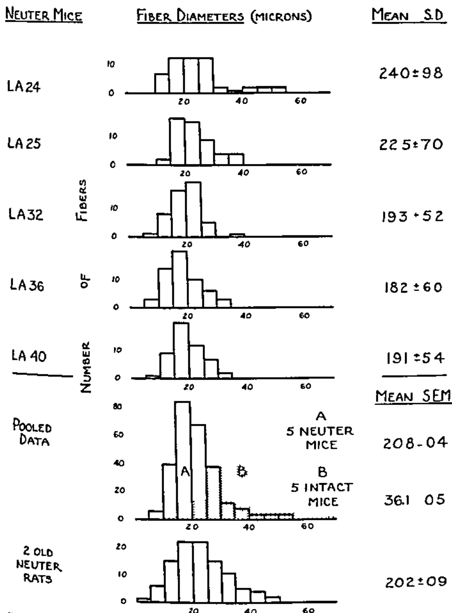



Fig 3 Histograms of muscle fiber diameters of the *levaor ani* of five neuter mice and two neuter rats castrated 45 days previously The data of the *neuter* mice (A) are compared to the data on *intact mice* (B) presented in figure 2

largement If these components are not replicated during this doubling of muscle volume one should expect volume dilution of organelles in each muscle fiber In fact, there appears to be little change in the relative distributions of the organelles This would imply that the formation of new organelles keeps pace with the expanding sarcoplasmic mass Whether or not such an increase occurs can not be proved or disproved easily by examining electron micrographs because of the prob-

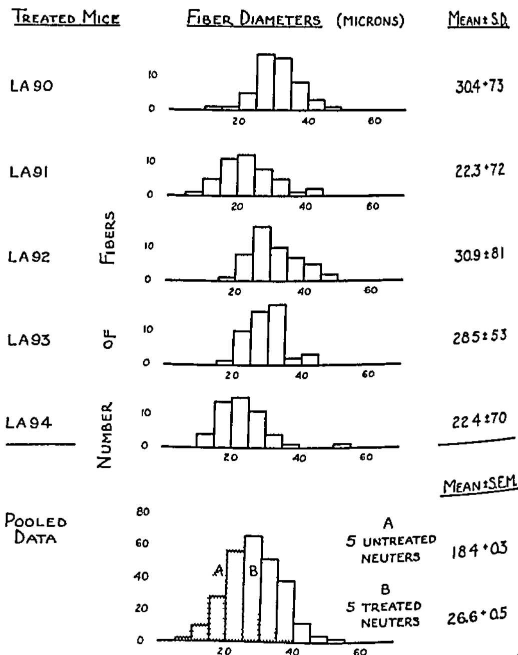



Fig 4 Histograms of muscle fiber diameters of the *levator ani* of five neuter mice treated eight days previously with 0 5 mg of Depo testosterone

lems of small sample size and non homogeneous distribution of the elements studied Leonard (50) has shown succinic dehydrogenase *concentration* to remain unchanged during enlargement of perineal musculature a fact conforming to a concept of replication of mitochondria during the process

## DISCUSSION

Kochakian has demonstrated ('64) that the enlargement of the *masseter* muscle of the guinea pig which occurs after administration of testosterone involves no increase in the quantity of DNA in the muscle This is true also both in the atrophy of the *levator ani* of the mouse following castration and in its reenlargement following testosterone treatment (Venable '66) The constancy of DNA is consistent with the lack of mitotic activity during these phenomena (Wainman and Shipounoff '41) The decrease and increase in size and weight of the muscle can be accounted

solely by a change in the average di ter of existing muscle fibers (Venable ) therefore these phenomena repre t a true atrophy and in reference to stable neuter condition a true hyper phy of the muscle cells This atrophy l hypertrophy is produced by a decrease l increase respectively of the usual ntractile proteins and supporting cyto- sm

With castration of the adult male mouse rat the rapid removal of testosterone of ticular origin apparently upsets dras ally the metabolic balance that main ns the muscle cells at their characteris- size The resulting metabolic imbalance es not kill cells but the loss of muscle iss becomes so rapid initially that the ual pattern of packed myofibrils is dis pted The apparent increase in the nount of sarcoplasm around the myo- nils results probably as much from a inning of myofibrils as from formation new sarcoplasm Many myofibrils par ularly those at the periphery are com etely lost others are only decreased in e The manner of dissolution of the con actile proteins and the sarcoplasm is un ear

Gori and Pelligrino (64) have presented preliminary report on the morphological pects of the atrophy of the *levator ani* rats following castration They note sosomes at the periphery of the fibers ar the nuclei In the present study lyso- mal like bodies are not observed at 22 ys post-castration but their presence rlier in the atrophy can not be denied seems improbable however that lyso- mal enzymes would be responsible for e degradation of myofilaments since it ould mean that lysosomes release spe fic enzymes into the sarcoplasm for pro- olytic digestion of the myofilaments and ssociated sarcoplasmic proteins One ould except that if the usual complement f acid hydrolases and proteases from a sosome would be released into the sarco- lasm there would be more drastic altera ons in muscle structure than what is ob- erved Indeed it is difficult to postulate ny result less than disruption of the cell ohn (64) has demonstrated that the loss f muscle proteins from muscles atrophy g from denervation has a non-enzymatic character despite the fact that lysosomal enzymes as measured by such means as hemoglobin degradation are in greater concentration

Gori and Pelligrino also report that large blocks of "undifferentiated sarco- plasma" appear sometimes as folds ex tending from the periphery of the fi bers that "will subsequently be phago- cytized by macrophages" Though such folds occur this method of removing sarcoplasmic material would likely be of little significance in the total reduction of size of the fibers since encounters of his- tiocytes or other phagocytes particularly in primary muscle bundles are rare The elimination of sarcoplasm occurs in what appears to be morphologically viable cells with neither massive destruction of all its elements nor accumulation of deposits of debris The method of autolytic breakdown of the sarcoplasmic elements remains for future investigation

It is significant that atrophy of the *leva tor ani* is self limiting Gori and Pelligrino (64) report a rapid 60% loss in weight of the muscle in male rats within one month after castration but that after this period the rate slows The muscle weights of *castrate* mice as reported herein drop at a similar rate nearing zero by 45 days By this time the muscles are one third their initial weights By 45 days the mus cle fibers show none of the alterations in the cytoplasm described as atrophic and are apparently in a stable condition There is no reason to doubt that the muscle is functionally active at this new decreased size since its fine structure can not be dif ferentiated from that of other white skel etal muscles A metabolic balance within the muscle fiber apparently has again been achieved that is commensurate with the hormonal balance of the *neuter* state Therefore testicular testosterone is not es sential to the existence of this muscle as a functional unit It is necessary only to maintain the muscle at the masculine size

It is surprising that the testosterone stimulated muscle can double its mass in nine days while maintaining a normal structural pattern in its muscle fibers No distinct areas of differentiating myoplasm appear Simply more and somewhat larger myofibrils fill the expanded fiber It is im

possible to characterize morphologically a myofibril either as a new or an old one This muscular hypertrophy is remeniscent of Morpurgo s early description of hyper trophy in the canine *sartorius* following forced exercise (1897) He showed the hypertrophy to result from an increased diameter of existing fibers by accumula tion of a greater number of myofibrils of similar size to those found in the unhyper trophied muscle It would seem that mus cle hypertrophy, whether resulting from exercise or from stimulation by testoster one is accomplished by similar processes though initiated by differing triggering mechanisms

Loring Spencer and Villee ( 61) pro vide evidence that testosterone may be linked with the metabolism of the *levator ani* via control of the activity of DPN cyto crome C reductase A high testosterone level by increasing this enzyme s activity would promote a greater abundance of ATP The availability of ATP could be the important rate limiting step in the syn thesis of sarcoplasmic and myofibrillar pro teins Assuming this to be true the con centration of ATP is probably influenced by multiple factors of which testosterone and exercise are only two Different fac tors apparently are expressed in varying degrees in different muscle groups Why testosterone expresses its effect most dra matically on the perineal musculature of the rodent and the muscles of mastica tion in the guinea pig remains a puzzle

From the evidence at hand the possi bility emerges that there is a common general process by which skeletal mus cle fibers synthesize and organize their unique cytoplasmic components the dif ferences in growth rate and final mass of different skeletal muscles depending on basic controls of energy producing metab olism

The data of Dreyfus et al ( 60) and Kruh et al ( 60) show a slow but distinct turnover of myofibrilar proteins in skeletal muscles of the rat and mouse These work ers further interpret their data as indicat ing a myofibril life span of 30 days in the rat and 20 days in the mouse Since myo-filaments may be synthesized and de stroyed continuously in adult animals hy pertrophy and atrophy can be thought of as an imbalance where the rate of either myofilament production or myofilament destruction predominates over the other

The fine structural patterns presented by myofilaments and myofibrils in a mus-cle fiber must, therefore be transient either being formed being temporarily stable or being broken down That each of these conditions in the adult has mor phologically distinct characteristics is open to question particularly if the rate of change is as slow as Dreyfus data would indicate Electron micrographs can demon-strate the rapid destruction of myofibrils as seen in the earlier stages of atrophy of the *levator ani* It is doubtful that they can be used alone to demonstrate slow destruction Myofilaments and myofibrils that are forming can not be identified with certainty on micrographs whether the syn thesis is rapid or slow though it is of at tempting to describe areas of slight disar rangement of myofilaments in these terms It will be necessary to combine other evi dence of synthesis with a particular mor phological pattern before interpretation becomes possible

The present study provides one hypothe sis for the structural manner in which the *levator ani* hypertrophies This hypothesis includes the assumption that myofibrillar patterns are not static and that new myo filaments are formed in their normal posi tions upon the sarcomere If ribosomal synthesis of proteins is represented which there is presently no reason to doubt the many of the granules in the sarcoplasm between myofibrils must be ribosomes As myofilaments accumulate on existing myo fibrils these myofibrils enlarge But not without limit because the sarcoplasmic reticulum divides the larger ones keeping the range of myofibril diameters within 0 5–1 5 μ Thus the number of myofibrils continues to increase yet very small myo fibrils — presumably new ones — seldom appear

The presence of the satellite cell of the muscle fiber as a consistent component of the *levator ani* representing over 5% of the nuclei enclosed within the glycocalyx of the muscle cell strengthens the accept ance of this cell type as one common to many skeletal muscles Since Mauro ( 61) first description of this cell in fr

rat skeletal muscle it has been re d to be present in cardiac muscle of species of crabs (*Chionoecetes opilio* *Charybdis japonica*) by Midsukami ) in the trunk musculature of the fish (*Myxine glutinosa*) and axolotl *ablystoma tigrinum*) by Flood (64) in the web muscles of the wings of t bats (*Eidolon helvum*) and several scles of white mice by Muir et al (65) frequency of occurrence has been mated in two instances other than the reported herein In the hagfish they resent two nuclei per 50 nuclei of mus fibers (Flood 64b) In the bat 10% nuclei associated with web muscle rs are satellite cells (Muir et al 65) ur and his co-workers have noted also it muscles of new born mice may have to 20% satellite nuclei associated with iscle fibers The satellite cells consist t presence in the *levator ani* in the same rcentage from animal to animal elimi ites any notion that it is only a wander g mononuclear cell caught in an unusual sition on a micrograph It is present roughout the usual life of white mice d rats (1 year) It is quite probable at the cells reported by MacConachie et (64) to exhibit mitotic activity in owing rat muscle are satellite cells as e has indicated and perhaps represent ells with myoblastic activity associated ith muscle development and maturation his study of the *levator ani* provides no nsight into the function of the satellite ell It apparently plays no roll in the trophy and hypertrophy of the *levator ani* n adult mice

ACKNOWLEDGMENTS

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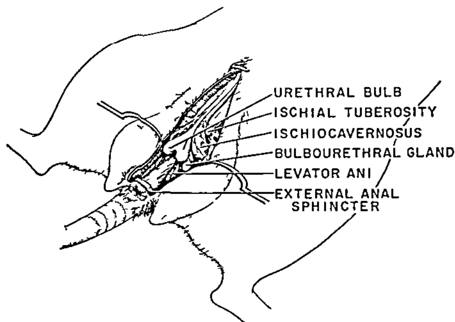
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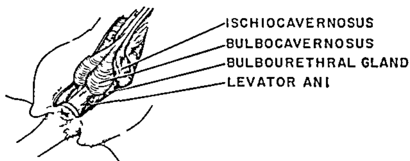
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## PLATE 1

### EXPLANATION OF FIGURE

5 The gross relationships of the *levator ani* of the intact mouse are illustrated here with the perineum dissected The upper drawing provides a view of the urethral bulb with the *bulbocavernosus* removed The lower drawing depicts the degree of dissection performed in preparation for the *in situ* fixation of the *levator ani* The dotted rectangle on the *levator ani* represents the sample used for morphological study


URETHRAL BULB
ISCHIAL TUBEROSITY
ISCHIOCAVERNOSUS
BULBOURETHRAL GLAND
LEVATOR ANI
EXTERNAL ANAL
SPHINCTER


ISCHIOCAVERNOSUS
BULBOCAVERNOSUS
BULBOURETHRAL GLAND
LEVATOR ANI

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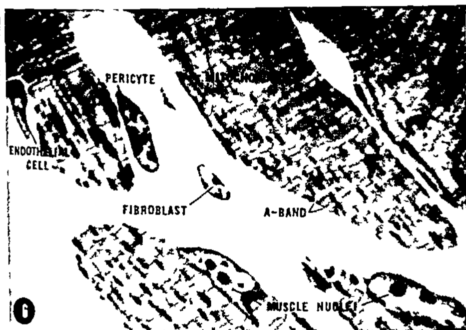
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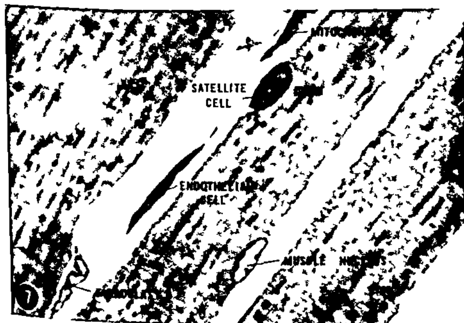
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PERICYTE
CELL
FIBROBLAST
A-BAND
6


SATELLITE
CELL
7

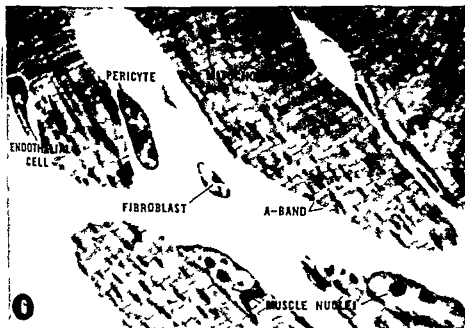
## PLATE 2

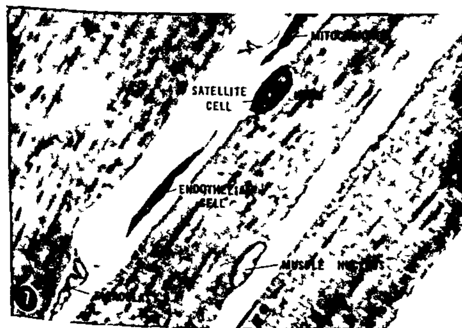
### EXPLANATION OF FIGURES

Epon sections three quarters of a micron thick of the *levator ani* from an *intact* mouse Stained with toluidine blue in borax × 1 700

6 The structure of the muscle is depicted at the limits of resolution provided by light microscopy Myofibrils pack the sarcoplasmic mass Mitochondria (dense sarcoplasmic masses) are scattered throughout the fiber Four nuclear types are noted Pericyte nuclei have darker chromatin patterns than muscle nuclei

7 A satellite cell appears in this section with the typical dark chromatin pattern Note the thin white line between the nuclear membrane of the satellite and the adjacent myofibrils This is a distinguishing characteristic of satellite nuclei in sections less than 1 $\mu$ in thickness Note that endothelial cells the fibroblasts (figs 6–11) are separated from the sarcoplasmic mass by a wider space No space is visible between muscle nuclei and the adjacent myofibrils


PERICYTE
CELL
FIBROBLAST
A-BAND
6


SATELLITE
CELL
7

## PLATE 3

### EXPLANATION OF FIGURES

Examples of cellular types and chromatin patterns in the *levator ani* Figures 8 9 and 10 are from *neuter* mice while figure 11 is from an *intact* mouse There are no obvious differences in sarcoplasmic structure of the muscle fibers between the two groups Nuclei are labeled by letter and number the letter being the initial of the cell type and the number indicating the chromatin pattern as ranked from 1 to 5 according to figure 1 × 1 500

8 The pericyte nucleus ($P_2$) has the typical chromatin pattern ranked 2 as does the endothelial nucleus ($E_2$) The muscle nuclei are typical ($M_4$) One fibroblast ($F_3$) is in view Erythrocytes fill the capillary

9 The pericyte fibroblast and endothelial cell nuclei all have chromatin patterns ranked 3

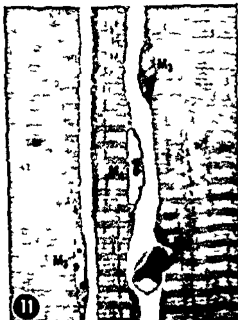
10 An intermediate form of pericyte satellite cell has a chromatin pattern ranked 2 ($P-S_2$) A motor end plate containing two terminal nerve endings appears at the lower right A Schwann cell satellite to a myelinated axon appears at the upper right

11 Various chromatin patterns of muscle nuclei Muscle nuclei with chromatin patterns darker and denser than the upper one ($M_3$) are rare Muscle nuclei with lighter chromatin patterns than those shown ranked 5 are common having a very thin layer of chromatin at the karyotheca and exhibiting a karyoplasm relatively free of chromatin strands (less than five thin strands)


F3
P2
E2
8


P3
F3
E3
9

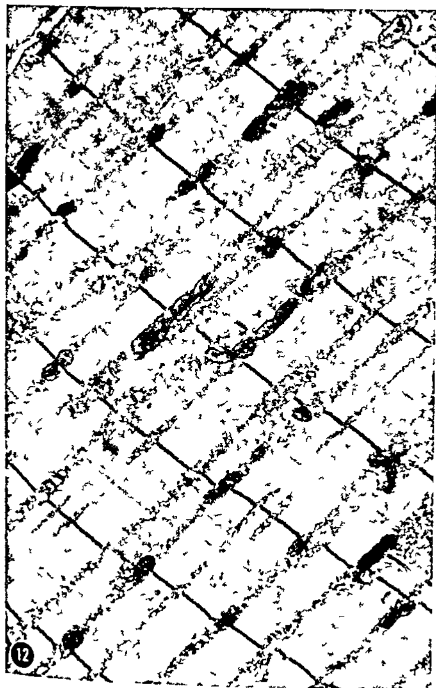

10


M3
M3
11

## PLATE 4

### EXPLANATION OF FIGURE

12 The *levator ani* of a *testosterone treated neuter* mouse The morphology is in basic respects no different from that found in *intact* and *stable neuter* mice A possible splitting of myofibrils in this hypertrophying muscle fiber is indicated by the incomplete division of many myofibrils by granular and tubular elements of the sarcoplasm No misoriented myofilaments are seen Sites of myofilament synthesis can not be located Glutaraldehyde-osmium fixation Lead citrate staining × 16 000


12

## PLATE 5

### EXPLANATION OF FIGURE

13 The *levator ani* from an *intact* mouse showing details of the organelles Mitochondria are scattered singly in the interfibrillar space The infoldings of the inner membrane form both cristae and tubules Small dark intermitochondrial granules are common The sarcoplasmic tubules of the longitudinal system are extensive and attached to the myofibrils at the Z lines (arrows) Triadic association of the longitudinally oriented tubules with the transverse tubules of the T system appears at all A–I junctions in this micrograph but may be missing at 25% of the usual sites Sarcoplasmic granules must represent both glycogen and ribosomes but a distinct differentiation of two types is not possible in this preparation Glutaraldehyde-osmium fixation Lead citrate staining × 40 000


TRIAD
TRIAD
13

## PLATE 6

### EXPLANATION OF FIGURE

14 A cross section of a satellite cell nested into a muscle fiber in the *levator ani* of a *neuter* mouse The nucleus shows much coarsely granulated chromatin and an ill-defined nucleolus A small Golgi complex lies to the left of the nucleus A few small mitochondria appear in the sparse granulated cytoplasm The plasmalemmae of the satellite cell and the muscle fiber are in close proximity There is no intervening glycocalyx except at the lateral margins Here (see inset) there is a separate glycocalyx for each cell More deeply along the interface of the two cells the two glycocalyces fuse thin and finally disappear Glutaraldehyde-osmium fixation Uranyl acetate-lead citrate staining × 10 000


14

## PLATE 7

### EXPLANATION OF FIGURE

15 A longitudinal view of a satellite cell in an intact mouse Only portions of the lips of the tangentially sectioned muscle fiber are seen encircling the satellite cytoplasm An intervening glycocalyx is double in some areas and fused in others The common close association of satellite cell muscle nucleus and capillary is illustrated Glutaraldehyde-osmium fixation Lead citrate staining × 10 000


PILLAR
15

## PLATE 8

### EXPLANATION OF FIGURE

16 Cells of the endomysium of the *levator ani* of a *neuter* mouse The pericyte displays two centrioles and an associated small Golgi complex The cytoplasm is sparse and granular like the satellite The nucleus characteristically shows thick clumps of coarsely granular chromatin at the karyotheca and in the karyoplasm The pericyte is encircled by a glycocalyx which is in close apposition but is not fused with the glycocalyx of the capillary The fibroblast lacks the glycocalyx Its nuclear chromatin pattern is lighter than the pattern in pericytes and its cytoplasm contains fewer free ribosomes The capillary is the type common to skeletal muscles Glutaraldehyde osmium fixation Uranyl acetate lead citrate staining × 16 000

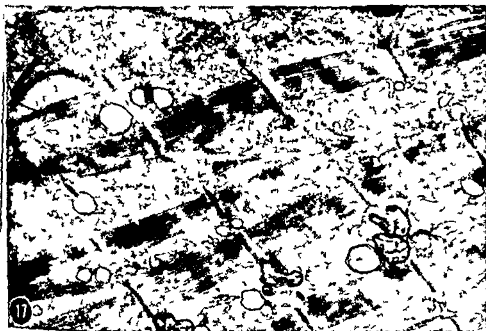
PLATE 9

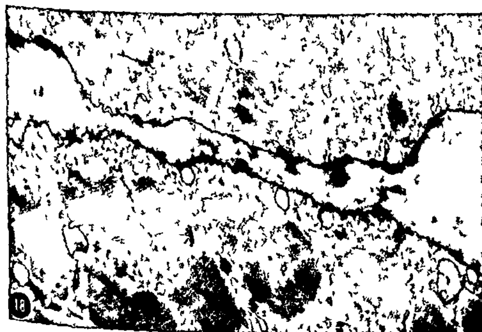
EXPLANATION OF FIGURES

*Levator ani* from a rat castrated 22 days showing sarcoplasmic changes associated with atrophy Osmium fixation Lead cacodylate staining × 20 000

17 A longitudinal section of the atrophying muscle fiber The myofibrils are separated by abnormal amounts of sarcoplasm Sarcoplasmic tubules are in disarray and apparently missing between some myofibrils Tubular attachments at the Z line are missing at many sites Myofilaments are frayed from the myofibrils Attachments at the Z-line appear more tenacious than attachments at the M line Dark granules appear (arrow) in the regions of overlap of thick and thin filaments

18 A cross section of the atrophying muscle fiber Dissassociation of myofilaments from myofibrils is evident Sarcolemma remains intact but wrinkled Sarcoplasmic tubules are sparse Arrow indicates dark granules corresponding to those indicated in figure 17


17


18

# dies on Guinea Pig Oocytes

## HISTOCHEMICAL OBSERVATIONS ON SOME PHOSPHATASES AND LIPID IN DEVELOPING AND IN ATRETIC OOCYTES AND FOLLICLES [1]

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*ABSTRACT* Normal guinea pig oocytes develop in an environment of AMPase within the granulosa cells of the wall the cumulus and the latter s projections that traverse the zona pellucida to terminate at the surface of the oocyte Oocytes in developing non vesicular and small vesicular follicles have an ATPase activity at their surface which appears to indicate specialized absorptive mechanisms confined to these early stages of development Acid phosphatase present in granules scattered throughout the cytoplasm of all developing oocytes may be evidence of a mechanism in this species whereby the oocyte hydrolyzes unidentified nutritional reserves

Histologically normal appearing oocytes in atretic vesicular follicles may show deviations from the normal histochemical pattern i.e clumping and subsequent loss of acid phosphatase activity or a band of AMPase activity at the periphery of the oocyte representing apparent enlargement of the terminals of projections of the cumulus These deviations may reflect disturbances of granulosa cell-oocyte transfer as a consequence of beginning atetric processes in the follicle wall By the eighth day of the estrous cycle the granulosa layer of many medium sized follicles has been invaded by a few long strands of nucleoside polyphosphatase positive thecal con nective tissue often carrying a small blood vessel. This early stage of atresia is apparently followed by shedding of the granulosa layer and hypertrophy of the thecal layer which begins the transformation of atretic follicles into interstitial masses

This histochemical study and a corre ted electron microscopic study (Adams nd Hertig 64) were initiated as prepara on for a similar study now underway on uman oocytes (Adams and Hertig 65) 'e hoped that by applying a variety of reparatory techniques in histochemistry nd electron microscopy to developing or tretic follicles and their contained oocytes 1 an available laboratory animal we ould be able to evaluate more efficiently imilar but much less easily obtained ma erial from human patients in the younger eproductive age The guinea pig was hosen because its relatively long estrous ycle (16–17 days) allows a controlled tudy of the sequence of events in the maturation of follicles and oocytes The histologic evidence for the maturation of a vesicular follicle within one estrous cycle in the guinea pig was reported in 1936 by Myers Dempsey and Young Their ob servations on developing vesicular follicles were used as the basis for this study

The application to guinea pig oocytes and follicles of histochemical techniques for a variety of phosphatases produced some results which vary considerably from those previously published for other mam malian species The results of our studies are reported here in order to add to the growing evidence of species differences and similarities in the cellular physiology of maturing oocytes and their follicle walls

In addition our histochemical studies of atretic oocytes indicate that certain pre cautions should be taken in the interpreta tion of the ultrastructure of any mam malian oocytes in vesicular follicles Even though the oocyte may show no histologic evidence of atresia and the follicle wall only minimal evidence of atresia our histochemical studies show that subtle changes frequently have occurred in the oocyte Such changes apparently involu

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tional undoubtedly also reflect alterations in the ultrastructure Therefore it seems hazardous to evaluate electron microscopic evidence of developmental vs degenerative processes in mammalian oocytes in vesicular follicles unless the follicular stage is known and unless the follicle can be fixed intact so that both the oocyte and the follicle wall can be carefully examined for the earliest evidence of atresia

## MATERIAL AND METHODS

Immature female guinea pigs purchased locally, were held until they began to establish a regular estrous cycle of 16–17 days This was confirmed by the periodic opening of the vagina for 2–3 days every 15–17 days Thereafter they were checked at least once each evening and morning for evidence of copulatory response indicating active estrus (heat) This was indicated when a female (1) attempted to mount other females (2) exhibited lordosis when stroked or placed with a male or (3) allowed a male to actively mount her (Young 61) Thirty animals 3–9 months of age were sacrificed by a blow on the head on various scheduled days after at least one and usually multiple recorded heats Ovaries were obtained on the first second fourth eighth twelfth and sixteenth days of the cycle and during heat (counting the estimated day of ovulation as day 1) Since ovulation occurs 8–10 hours after the onset of heat those animals to be sacrificed for the recovery of matured follicles were sometimes observed constantly during the evening of expected estrus in order to time the onset of heat and thus to determine the time of subsequent sacrifice

One ovary from each animal was fixed in Bouin s fluid for histologic survey of follicular and oocyte development These ovaries were sectioned at 7 μ and every third section was serially mounted and stained with hematoxylin and eosin The other ovary was fixed overnight in either chilled formol calcium (Baker 51) or formol phosphate with 7.5% sucrose (Holt 61) After washing for one to one and one half hours in running water it was placed in a freshly prepared solution of gum sucrose for three hours and was then rapidly frozen in a beaker of isopentane precooled to − 70°C in a bath containing dry ice and acetone These ovaries were serially sectioned in a cryostat at 10–12 μ — each section was mounted separately on a precooled gelatinized slide and dried briefly on a warm plate at 35°C The slides were quickly examined with a phase microscope to record those serial sections containing oocytes and then were stored overnight at 4°C By this means serial slides of an individual oocyte could be used for several different techniques the following day

Incubation media for the nucleoside phosphatase activities were freshly prepared according to the method of Wachstein and Meisel (57) using 0.5 mg of substrate per ml of medium at pH 7.2 Adenosine triphosphate (ATP) adenosine diphosphate (ADP), adenosine 5-phosphate (AMP), and sodium glycerophosphate (NaGP) were the substrates used at this pH Sodium glycerophosphate served as a control for the specificity of the nucleoside phosphatases An additional slide was run as a water blank control in the complete medium without substrate Sections were incubated 25 minutes at 37 C then thoroughly rinsed in distilled water and placed in dilute ammonium sulphide 1–2 minutes After a thorough wash in water they were mounted in glycerine jelly

Slides for acid phosphatase activity were incubated for one hour according to the Gomori method at pH 5.0 as reported by Becker et al (60) using sodium glycerophosphate as substrate Sodium glycerophosphate was also the substrate for alkaline phosphatase activity performed according to the Gomori method at pH 9.4 (Pearse 60)

Additional sections were stained by Sudan Black for lipid and occasionally others were post fixed on slides in Rossman s fluid and stained by the periodic acid Schiff method for glycogen

## OBSERVATIONS

### *Normally developing follicles*

#### *Histologic description*

*Primordial and non vesicular (primary) follicles* At all stages of the estrous cycle primordial and small primary follicles are found at the periphery of the ovary (fig 2) Primordial oocytes measuring 20–25 μ in

ieter surrounded by a single layer of ned primitive follicular cells contain ntral nucleus with clumped chromatin 3) Ioannou (64) reported that this itracted stage of development follows large diplotene stage and becomes pre inant in resting oocytes of guinea pigs r than 60 days These small follicles in a narrow band of dense cortex just eath the subepithelial tunica albuginea

initial evidence of development to the nary follicle stage is an increase in the nber of follicle cells as they become oidal The oocyte begins to enlarge in meter and its nucleus becomes vesic r The single layer of cuboidal cells h prominent nucleoli are not observed mitosis Once the cuboidal cells of the licle wall begin to become double lay d however mitoses are frequently seen g 3) During the development of the iltilaminar non vesicular follicle the yte grows markedly in diameter the nulosa wall becomes multilayered the ia pellucida is formed and the theca erna differentiates In addition the fol le is carried deeper into the ovary where lies within the cortex usually adjacent an interstitial mass

*Vesicular (secondary) follicles* The histo gic survey of our serial sections of ovaries amined on the first second fourth hth twelfth and sixteenth days follow g an observed estrus indicates a matura n of the vesicular follicles during the riod of one estrous cycle These observa ns are in agreement with those reported Myers Young and Dempsey (36) The owth and atresia of guinea pig follicles schematically illustrated in figure 1 ich is reprinted from Everett (61)

As Myers et al reported only a few of e many small vesicular follicles found each ovary at about the fourth day of e estrous cycle are destined to produce e 1–3 mature follicles of the sixteenth y (Although similar small follicles are esent on later days of the cycle all ap rently undergo atresia without maturing ig 1)) Developing follicles character tic of the fourth day of the cycle have an centric crescent shaped antrum sur unded by a multilayered granulosal wall central granulosal mass forming a cumu s around the oocyte and a broad theca interna layer (figs 4 5) They measure 0 2–0 3 mm in diameter to the periphery of the granulosal layer the external bound ary of theca being hard to define in any follicle Mitoses are prevalent in the cumulus and throughout both granulosa and theca interna layers The oocyte is spherical measuring 65–70 μ in diameter (fig 5) It has a vesicular nucleus which is central in the smaller follicles and often more eccentric in the larger ones of 0 3 mm In Bouin s fixed material the sub stance of the zona pellucida is not pre served and is seen merely as a space trav ersed by projections from the inner layer of the cumulus

By the eighth day normally developing follicles measure 0 6–0 7 mm in diameter have a thick wall of granulosal cells loosely attached to each other and a weblike cumu lus attached at several points to the granu losal wall (fig 6) Many mitoses can be seen in both the granulosal and thecal layers The oocyte has an eccentric nu cleus with a large vacuolated nucleolus (fig 7) Its cytoplasm appears mottled due to lipid droplets having been dissolved out in the histologic preparation

By the twelfth day of the cycle in each ovary there are usually 1 to 3 follicles

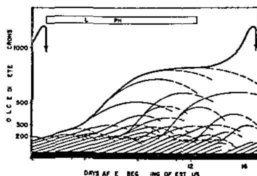



Fig 1 A schematic representation of the fol licular cycle in the guinea pig The heavy solid curve represents the diameters of the largest follicles recalculated from the data of Myers Young and Dempsey ('36) The arrow point indicates ovulation The other solid curves and broken lines represent impressionistically the growth and atresia respectively of other groups of follicles that are not ordinarily destined for ovulation

This diagram of the development and atresia of secondary follicles in guinea pig oocytes is reproduced from Everett (61)

measuring 0 8–0 9 mm whose peripheral granulosal layer is often luteinized or eosinophilic Lacunae of fluid begin to collect forming a spongy zone at the base of the cumulus — actually a septum traversing the follicle (fig 9) In contrast to the follicular cells the nuclei of the cumulus cells are widely separated by indistinct cytoplasm The innermost row of these cells is forming a corona radiata Their nuclei lie distal to the oocyte in an elongated cytoplasm which is adherent to the zona pellucida (fig 8) Their delicate cytoplasmic processes project through the zona to the oocyte membrane

During the sixteenth day the maturing follicles measure 1 0 mm or more and the granulosa cells become strikingly eosinophilic as luteinization continues The lacunae of fluid in the base of the cumulus are larger (fig 11) Just before the beginning of heat the eccentric nucleus of the oocyte develops a network of pale fibrils and a dense basophilic chromatin mass (fig 10)

During heat the oocyte forms the first meiotic spindle (fig 12) extrudes a polar body and begins to form the second meiotic spindle The cells of the cumulus dispserse and a coagulum is formed which loosely attaches the oocyte and surrounding corona radiata to the follicle wall (fig 13) The follicle expands to over 1 mm in diameter Only a very few ovaries examined during heat contained any normal appearing secondary follicles of any size other than the 1 to 3 matured follicles destined to ovulate

## HISTOCHEMICAL OBSERVATIONS

In contrast to oocytes in later stages of development the cytoplasm of most resting primordial oocytes has been somewhat difficult to preserve in frozen sections without distortion Thus we have been unable to assess accurately the results of histochemical procedures in the primordial oocyte itself Nevertheless no resting primordial oocyte appeared to contain any cytoplasmic enzymatic activity by any of the methods employed

The superficial cortical cells as well as those of the tunica albuginea in the guinea pig ovary contain an *adenosine monophosphatase* activity indistinguishable from that seen in the thin single layered primordial follicle cells (fig 14) In developing early primary follicles AMPase activity is present at the periphery of the cuboidal follicle cells but is most prominent in the area between the inmost layer of these cells and the oocyte (fig 15) From electron microscopic studies on guinea pig oocytes in these stages this area has been observed to contain a concentration of broad interdigitating projections arising from follicle cells (Adams and Hertig '64) In later stages of multilaminar non vesicular follicles AMPase activity is seen in these projections that now traverse the developing zona pellucida to terminate at the cellular membrane of the oocyte As intercellular spaces between granulosa cells become more prominent AMPase becomes localized at the periphery of each cell

In small secondary follicles developing on the fourth day of the estrous cycle adenosine monophosphatase remains prominent at the margins of all granulosal cells (fig 16) In these follicles just beginning to form an antrum the enzyme is most active immediately peripheral to the oocyte where it again delineates the mesh work of granulosal projections surrounding the zona the delicate projections that traverse the zona, and the blunt terminals of these same projections at the oocyte membrane (fig 17) By the eighth day stage there is no longer a dense band of activity peripheral to the zona (fig 18) and in addition AMPase in the entire granulosal layer becomes somewhat less prominent (fig 19) The projections of the follicle cells that traverse the zona become more delicate and widely spaced but retain their AMPase activity until the onset of heat at which time it is lost (figs 18 20 21) Occasionally cytoplasmic granules of AMPase activity are present in the oocyte on the twelfth and later days of follicular development

In double or multilayered non vesicular follicles a fuzzy rim of *adenosine triphosphatase* activity becomes prominent at the periphery of the oocyte (figs 22 23) With adenosine diphosphate as a substrate however activity in this area is only rarely seen (fig 24) The rim of ATPase activity is still present around oocytes in 0 2–0 mm follicles of the four day stage (fig

26) but we have never seen it in nor ly developing oocytes in larger follicles ater development Oocytes in follicles similar size (0 2–0 3 mm) present at er stages of the estrous cycle also show ATPase activity (fig 59)

A less specific *nucleoside polyphospha e* activity produced with both ATP and P develops in cytoplasmic granules in ytes of the larger primary follicles g 22) These remain small and dif ely distributed in the four-day follicles g 26) but subsequently become less merous By the twelfth day those seen th ATP as a substrate appear coarse and mewhat more peripherally located (figs 28) whereas with ADP as a substrate e granules are smaller more numerous d more evenly distributed in the cyto asm (fig 29) Nucleoside polyphospha se activity with both ATP and ADP as bstrates is also present in the vascula ure and supporting cells of the theca in rna of all follicles (fig 25) in interstitial asses and in the supporting connective ssue of the ovary It is less prominent in e tunica albuginea and is absent in the rosal epithelium

Sodium glycerophosphate as a substrate t a similar neutral pH produces no activ ty in oocytes of any stage but in the theca nterna layer produces a slight reaction ocalized similarly to an alkaline phospha ase (fig 60) In sections reacted as a water blank the granulosa and theca cells frequently contain a single black granule which is interpreted as inorganic phos phate reaction produced in some unidenti fied lipid The oocytes are always free from these granules

In the small primary follicle stage the oocyte begins to develop a few cytoplasmic granules of *acid phosphatase* (fig 30) Such granules are distributed throughout the cytoplasm of oocytes in developing vesicular follicles of all sizes (figs 32 33 35) Cells of the cumulus also have a fine dusting of cytoplasmic activity Activity in macrophages invading the theca interna is often seen in larger vesicular follicles During estrus there is a faint reaction in the granulosal cells of matured follicles and strongly reactive macrophages begin to invade this layer as well (fig 34)

Water blank controls frequently show a granule of precipitate in the granulosa and thecal layers as well as in the serosal surface similar to that seen at neutral pH (fig 31)

As the primary follicle enlarges and moves deeper in the ovarian cortex it be comes associated with the *alkaline phos phatase* positive cells of the interstitial masses (fig 36) Reactive theca interna cells begin to appear around the primary follicle (figs 36 and 37) In the maturing vesicular follicles alkaline phosphatase activity in this layer continues to be pres ent (fig 55) but does not become so prom inent as that seen in the hypertrophied theca of atretic follicles (fig 61)

*Sudanophilia* is diffuse in oocytes of pri mary follicles and probably represents mitochondrial staining (fig 38) Fat vacu oles develop in the small vesicular follicles characteristic of the fourth day stage (fig 39) These vacuoles increase rapidly in number and fill the cytoplasm as the nu cleus becomes eccentrically located (figs 40 42) Fine sudanophilic droplets are present in the peripheral layers of the granulosal wall of the mature 16-day fol licle and increase in prominence during heat (fig 41) Previous to this only a single small granule of sudanophilia was visible in some cells of the cumulus (fig 42) and granulosal wall These were in terpreted to be the same granules seen in the water blank slides of neutral and acid phosphatases (fig 31) The cells of the thecal layer are finely sudanophilic in the secondary follicles

*Glycogen* confined to the cells of the cumulus only is prominent in mature fol licles of the sixteenth day During heat it is seen throughout the coagulum contain ing the oocyte

### *Atretic follicles and oocytes*

Normal and abnormal appearing pri mordial and primary follicles have been observed throughout the estrous cycle In the primordial oocyte it is difficult to dis tinguish atretic processes from nuclear or cytoplasmic distortion that may be caused by preparatory technique Occasionally primordial sized oocytes with contracted" nuclei are seen in follicles whose cells are cuboidal and are prematurely undergoing

mitosis In primary follicles extreme eccentricity of the nucleus and irregularly arranged granulosal layers are evidence of atresia (fig 43)

Early in the estrous cycle as new small vesicular follicles develop the follicles from the previous cycle are in various stages of atresia leading to the formation of new interstitial masses (figs 46–50) This transition proceeds gradually by the sloughing of the granulosal layer into the cavity and the hypertrophy of the theca interna followed by the organization of the antrum The thecal hypertrophy results in an interstitial mass in whose central cavity a degenerating oocyte can often be seen (figs 49 50) These oocytes usually have undergone cleavage (fig 49) or fragmentation but occasionally they may show bizarre nuclear changes such as large astral rays associated with clumps of basophilia (fig 82) or multiple micronuclei (fig 83) These degenerating oocytes contain large deposits of lipid (fig 69) Although their acid phosphatase activity has previously been lost (fig 75) a cytoplasmic substance occasionally is found that gives a black precipitate with the acid phosphatase reaction and also with its water blank control (figs 76 77) Such a reaction is characteristic of the deposition of insoluble salts Acid phosphatase (fig 75) and sudanophilia (fig 69) are seen in the macrophages of the organizing antrum As the thecal layer hypertrophies to form an interstitial mass its alkaline phosphatase (figs 61 62) and lipid content becomes more pronounced (figs 75 39) and acid phosphatase (fig 32) appears faintly in a perinuclear distribution

The atretic processes which overwhelm the great majority of vesicular follicles may become apparent at any stage of their development At all stages of the cycle the oocytes in small vesicular follicles may show a precocious eccentricity of the nucleus (fig 44) The granulosal layer or the cumulus is often abnormally thin in spite of the presence of mitotic figures in these layers (fig 44) In these small follicles there also may be sloughing of granulosa cells into the cavity (fig 45) The cells of the granulosal layer retain their AMPase activity until they are shed (fig 68) In the oocyte acid phosphatase is occasionally seen in paranuclear clumps rather than in dispersed granules (fig [illegible]) Oocytes in non developing small follicles often retain their peripheral rim of ATPase (fig 59)

Prominent on the eighth day of the cycle in medium sized follicles that seem to be otherwise developing normally is an invasion of the granulosa wall and the cumulus by one or two long strands of connective tissue cells from the theca interna (figs 51 52) These strands which usually carry a small blood vessel (fig 54) traverse the cumulus but do not invade the oocyte or zona Histologic study of serial sections through many follicles in the eighth day of the cycle reveals that only a few medium sized follicles appear to escape this form of atresia The nucleoside polyphosphatase activity (with ATP or ADP) of the invading cells allows one to identify quickly this atretic process in scanning an ovary of this stage for normally maturing follicles (fig 53) The invading cells are not the alkaline phosphatase positive theca interna cells (fig 55)

After the twelfth day of the cycle the two or three follicles destined to ovulate from each ovary are usually recognizable histologically by the increased eosinophilia of the granulosal layer This distinguishes them from other non maturing follicles of similar or smaller size which may not yet show evidence of atresia Most of the non maturing follicles however do show beginning or widespread sloughing of the granulosal cells into the antrum or hypertrophy of the theca or both (fig 46) The sloughed granulosal cells are rounded and AMPase negative (fig 63) but those still within the wall retain an active cytoplasmic rim In spite of this indication of involution mitoses still occur in the wall or the cumulus (fig 46) In the granulosal layer of some sloughing atretic follicles occasional strands of nucleoside polyphosphatase positive thecal elements are present This finding is the residual evidence of the earlier invasive (8th day) stage In other involuting follicles of the twelfth to sixteenth days of the cycle showing more advanced shedding of the granulosal layer a broad stalk of thecal tissue projects into the cavity near the base of the cumulus (fig 56) This again is interpreted as a

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With the onset of heat most oocytes in non-ovulating follicles of varied sizes and sta es of atresia begin to form spindles and to extrude a polar body (figs 78–80) Some undergo cleavage to morula like forms (fig 81) Occasionally this abnor mal nuclear maturation is seen in oocytes in small follicles that do not yet show cytologic evidence of atresia (fig 78)

At all stages of the cycle there are a variable number of normal appearing fol licles which are not developing synchron ously with those follicles whose growth was initiated soon after estrus They vary greatly in size and may be derived from small follicles arising throughout the cycle (fig 1) It is difficult to determine how many of them will eventually contribute to the pool of interstitial masses seen in the ovary throughout the cycle

## DISCUSSION

### *Normally developing oocytes and follicles*

1 *Nucleoside monophosphatase (AMPase)*

Although the function of this enzyme in the economy of the guinea pig oocyte is un known it is always present at the imme diate periphery of the resting and maturing oocyte Its presence in the follicular projections that terminate at the oocyte mem brane suggests that there may be special ized routes for various substances reaching or leaving the developing guinea pig oocyte These projections may transport material synthesized in the cells of the cumulus and delivered directly to the surface of the oocytes During estrus the loss of the activity of this enzyme in the projections traversing the zona pellucida correlates with the time of dispersal of the outer layers of cumulus cells It is an indication that these cells rather abruptly are no longer required for the further maturation of the oocyte The reactivity of the entire granulosal layer may indicate some role in the production or regulation of the fol licular fluid Anderson and Beams ( 60) the first to study the ultrastructure of the guinea pig oocyte postulated that the fol licle cells of the cumulus synthesize a nu trient which is liberated into the zona pellucida and reaches the oocyte by diffu sion or pinocytosis

Other mammalian species have not to our knowledge been reported to contain AMPase in the granulosal wall of developing follicles McKay et al ( 61) reported the presence of this enzyme only in the stroma surrounding follicles in the human ovary In the mouse Novikoff et al ( 61) reported its presence only in the germinal

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epithelium Arvy (60) reported its presence in the rabbit ovary at the periphery of the oocyte between the zona pellucida and the surface of the oocyte Although she did not report the visualization of the projections of the cumulus cells in the zona it is possible that the rabbit may be similar to the guinea pig in the localization near the oolemma Her report however described a granulosa rich only in alkaline phosphatase

2 *Nucleoside polyphosphatases*

The strong reaction for this class of enzymes using either ATP or ADP as a substrate seen in many structures of the guinea pig ovary is similar in distribution to that reported by Novikoff et al (61) in the mouse ovary and by Arvy (60) in the rabbit In addition guinea pig oocytes within large primary follicles or small secondary follicles contain numerous fine cytoplasmic granules of activity In oocytes of larger follicles only a few coarse granules were seen The precise localization of these intracytoplasmic granules is unknown and awaits histochemical electron microscopic study

A somewhat more specific reaction namely for nucleoside triphosphatase was seen in a narrow fuzzy halo at the periphery of the oocyte during the primary and very early secondary follicle stages It is during these early stages that microvilli begin to protrude from the oocyte membrane into the zona pellucida and a complex system of peripheral organelles is being differentiated within the oocyte cytoplasm (Adams and Hertig 64) Therefore we believe this nucleoside triphosphatase may well be localized on or near these microvilli Thus in contrast to the AMPase of the follicular projections this enzyme may be involved in the absorptive capacity of the oolemma for substances perhaps filtering across the zona pellucida from the follicular fluid Moreover the presence of this enzyme at the periphery of the oocyte in its early stages of development and its absence as the follicle enlarges beyond 0 3 mm diameter appear to reflect changes in the absorptive mechanism during development of the oocyte Our unpublished electron microscopic data show that microvilli are present in maturing oocytes as late as the twelfth day stage This is however long after the disappearance of ATPase and may represent altered function of the microvilli In mouse ovaries Novikoff et al (61) observed that with ATP as a substrate there was intense staining of the zona pellucida whereas with ADP as a substrate the zona of an occasional oocyte was negative These authors also postulated a correlation between this activity and the presence of microvilli on the surface of the oocyte and interpreted the activity as indicating the presence of either a polyphosphatase or the presence of both ATPase and ADPase In the guinea pig oocyte reactivity with ADP as a substrate was only rarely observed and even the reactivity with ATP appeared to be dependent on a very brief fixation time This particular localized sensitivity to technical procedures may indicate the presence of an enzyme or enzymes in the oolemma different from nucleoside polyphosphatases elsewhere in the ovary The latter enzymes withstood longer fixation times in our hands

3 *Acid phosphatase*

This enzyme is present as discrete cytoplasmic granules in all developing guinea pig oocytes We have been unable to correlate the size or number of reactive granules with the stage of development The variation in size of the granules has seemed to depend on subtle differences in technical preparation since all normal oocytes in any one ovary regardless of their stage of development contain similar sized granules Therefore we have attributed this variation to the degree to which the organelles containing the enzyme became swollen during the fixation and freezing processes

Although the presence of this enzyme in a variety of cells has been correlated with the presence of lysosomes — membrane bound structures containing a variety of hydrolytic enzymes — our previous el tron microscopic study of the early dev oping guinea pig oocytes did not reveal an organelle morphologically recognizable to us as a lysosome We did however find large vesicles containing a slightly den substance which might from the point of view of cytoplasmic distribution be p

e sites for the ultrastructural localiza 1 of this enzyme These were expanded icles of rough endoplasmic reticulum l were postulated to be areas for pos le storage or synthesis of material hin the oocyte (Adams and Hertig 64) tochemical observations on an electron croscopic level will obviously be required localize the intracytoplasmic site of ac ity The function of acid phosphatase thin the developing oocyte is unknown t its presence could indicate this hydro- ic enzyme is required for the utilization some nutritional reserve Although in e guinea pig lipid appears to be the prin pal material stored in the cytoplasm e acid phosphatase granules can be seen light and phase microscopy to be dis ict structures separate from the lipid oplets This enzymatic activity appears developing oocytes before the stage hen fat droplets can be identified histo- gically

Acid phosphatase is also present in acrophages in the thecal layer of the fol le Just before ovulation considerable umbers of these phagocytic cells invade e granulosal layer of the matured follicle heir presence here may be related to the osening of this layer in preparation for e penetration of thecal vessels and its onversion into a corpus luteum

Arvy ( 60) reported that the oocyte and ranulosal layer in rabbit ovaries were ich in acid phosphatase McKay et al 61) studying the human and using ace one fixation and paraffin sections reported he presence of acid phosphatase in ocytes granulosa and theca in preantral follicles and in the granulosa and thecal ayers of large Graafian follicles Our own recent unpublished studies of similar hu man material in formalin fixed frozen sec tions show acid phosphatase activity in the follicular wall but not in the oocyte It is now recognized that an acetone paraffin technique allows diffusion of acid phos phatase activity Lobel et al ( 61) re ported the absence of acid phosphatase in healthy follicles and in all oocytes of rat ovaries

4 *Alkaline phosphatase*

This enzyme was never found in the oocytes nor in the granulosal layer of developing follicles Its presence in the thecal cells of non vesicular and vesicular follicles and in the interstitial masses may be involved in some unknown way in the production of estrogen

Studies of other mammalian species have indicated a variety of combinations of alkaline phosphatase localizations as re ported in a review by Jacoby ( 62) Both the hamster (Knigge and Leathem 56) and the rabbit (Arvy 60) have a granu losal layer rich in alkaline phosphatase with lesser amounts or absence of activity in the theca Most other species however including humans (McKay et al 61) have been reported to contain alkaline phosphatase in the thecal layer (Jacoby 62) We know of no other species that has been reported to have a combination similar to that reported here for guinea pigs i e a strong nucleoside monophos phatase activity in the granulosa com bined with a strong alkaline phosphatase activity in the thecal layer

5 *Sudanophilia*

Fat droplets appear in the guinea pig oocyte in developing follicles at about the fourth day of the cycle De Geeter ( 54) who studied some histochemical aspects of oocytes and fertilized ova in guinea pigs rats and rabbits suggested that lipid appears to be the main nutritional storage reserve in guinea pig oocytes In this con nection it is of interest that lipid droplets appear soon after the stage when our pre vious electron microscopic observations on primordial and primary follicles led us to conclude that the oocyte had become equipped for the absorption utilization and intracellular transport of material deliv ered to its surface membrane (Adams and Hertig 64) The presence of fat droplets in oocytes of the sow horse cat and dog were noted by Jacoby ( 62) who reviewed reports of their possible source from the yolk nucleus and/or from cells of the cumulus

A faint sudanophilia is present in cells in the thecal layer of developing follicles It also appears in the peripheral layers of the granulosa in matured follicles where it may be related to the "luteinization" of this layer and its subsequent transforma tion to a corpus luteum The true relation

ship of sudanophilia in the follicle wall to steroid production and/or secretion is uncertain since we did not attempt to demonstrate steroid 3B ol dehydrogenase activity on these formalin fixed frozen sections The relationship of sudanophilia to other indirect histologic tests for hormone production is discussed fully by Jacoby ( 62) who reviewed a variety of reports on several species

6 *Glycogen*

Glycogen deposits appear in the cells of the cumulus just before heat as well as in the coagulum during heat This may be evidence of the cessation of the transport to the oocyte of glycogen precursors from the cells of the cumulus and the consequent deposition of glycogen in these cells The appearance of glycogen in these areas occurs at the same time that the follicular projections lose their adenosine monophosphatase reactivity In the guinea pig the cells of the outer layers of the cumulus are dispersed during heat and those of the corona radiata are detached from the ovum soon after ovulation (Hunt and Chang 64)

*Atretic oocytes and follicles*

There are three morphologic aspects of atresia those involving the oocyte the follicle and the combination of these two in relation to the cycle In primordial primary (non vesicular) and very small secondary (vesicular) follicles the earliest sign of abnormality is frequently detected by the eccentric location of the egg nucleus This would seem to indicate that at these stages abnormal differentiative processes are initiated within the oocyte itself and are independent of the stage of the cycle Hisaw ( 47) observed that the arrangement of the granulosa of a primordial follicle is apparently under the control of the ovum Whether or not abnormal oocytes are indeed the cause of the poorly developed or disorganized granulosa layers seen in many of our non maturing primary and small secondary follicles has not been clarified by any of the present observations

The phase of the estrous cycle seems to be related to the atretic processes that overwhelm the great majority of vesicular follicles whose apparently normal development is initiated soon after estrus This cyclic atresia appears to be initiated in the follicle wall with secondary effects on the oocyte Initially this form of atresia can be seen in ovaries examined on the eighth day of the cycle when an invasion of the granulosal layer and the cumulus by delicate strands of vascularized connective tissue is taking place It is easily observed in nucleoside polyphosphatase preparations of moderately large follicles but less easily seen in sections stained by H and E The invasive process appears to be evanescent since examination of ovaries on later days of the cycle shows no continuation of the process Instead of the latter beginning or advanced shedding of granulosal cells is seen in follicles with residual strands of invasive thecal elements in the granulosal layer This phenomenon of thecal invasion appears to be the earliest indication of a selective atretic process which is possibly under endocrine control This interpretation is based on two observations (1) that these invasive stages are seen in many follicles that had apparently begun their development normally and (2) that the number of 0 6–0 7 mm follicles that had escaped this invasion by the eighth day of the cycle approximates the number of follicles which by the twelfth day show obvious evidence of maturity i e eosinophilia of the granulosal wall Thus the critical period when the follicles destined to undergo atresia are separated from those destined for maturation has been reached by the eighth day — well before the twelfth day when Dempsey ( 37) reported that some guinea pig follicles are competent i e capable of being ovulated A causal relationship between the vascularized connective tissue invasion and the subsequent shedding of the granulosa and the hypertrophy of the thecal layer in atretic follicles has not been clearly defined by this study In fact we do not know whether this invasion is cause of or a response to a subtle beginning of atresia In a discussion of causal factors of atresia Ingram ( 62) observed that many experimental procedures which affect the rate of atresia also involve some alteration of the supply of gonadotrophin In commenting on the possible control by the pituitary of the variation of atretic processes he observed that the "vascular

individual follicles may also participate se follicles provided by a relatively poor od supply (and hence a low level of nadotropin) probably being the first to ome atretic

By our criteria the presence of even one two sloughed granulosa cells in any ially sectioned follicle indicated atresia espective of the presence of mitotic fig s As previously observed by Schmidt (2) the stimulus to growth in the fol le wall revealed by mitotic figures con ues despite rather widespread slough deed mitotic figures in the cumulus can ten be found in follicles with advanced anulosal shedding The abnormal ap arance of nucleoside polyphosphatase in e cytoplasm of the basal granulosal yers often appeared to be an early evi nce of atresia and to be correlated with edding of the more distal layers into the vity This breakdown of the granulosal yer was accompanied by neither the ap arance of acid phosphatase as was re orted by Lobel et al (61) in the rat nor e dramatic increase in lipids reported a variety of mammals by Guraya and reenwald (64)

In many follicles with sloughing gran losa cells the oocyte often appears nor nal histologically as observed previously y Rowlands (56) In some of our fol icles with only minimal atresia however he contained oocyte though appearing ormal histologically showed histochem cal evidence of abnormality The nature f these aberrations appears to indicate he sensitivity of the oocyte to the integrity f its follicular environment In AMPase preparations the visualization of the ap parent fusion of the terminals of the gran ulosal projections at the periphery of the ocyte appeared to be histochemical evi dence of *interruption* of granulosa cell oocyte transfer The clumping of acid phosphatase activity in the cytoplasm of oocytes in follicles with early atresia may also reflect the lack of orderly transport to the oocyte of some nutrient supplied to it from the environment The apparently rapid disappearance of this activity in the oocyte as the follicle continues to degen erate indicates that this hydrolytic enzyme is not involved in the dissolution of this cell The enlarged lipid droplets in degenerating oocytes may be an indication that this nutritional reserve can no longer be utilized

Just before ovulation most oocytes of non-ovulating follicles contain matura tion spindles with or without polar body formation This phenomenon and the subsequent development of micronuclei morula like formation and bizarre nuclear transformations can be followed in degen erating oocytes as the follicle becomes transformed into an interstitial mass Harman and Kirgis (38) postulated that the stimulus for this abnormal maturation division in the guinea pig is related in some way to the presence of sloughed or necrotic granulosa cells On the other hand our observation of the presence of spindles in oocytes in occasional small fol licles not yet showing cytologic evidence of atresia supports suggestions made in dis cussions by Hammond (61) Chang (61) and Pincus (61) These authors postulate that such spindles may form at about the time of ovulation due to release from an unidentified factor that inhibits matura tion and cleavage

ACKNOWLEDGMENT

The authors gratefully acknowledge the participation in this project by Mr Arlo Collins who faithfully recorded his ob servations on the estrous cycles of these guinea pigs In addition thanks are due to Mrs Audrey Hadfield for photographic assistance and to Mrs Millicent Snow for secretarial help

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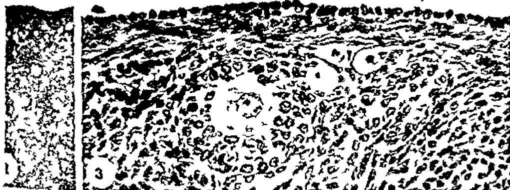
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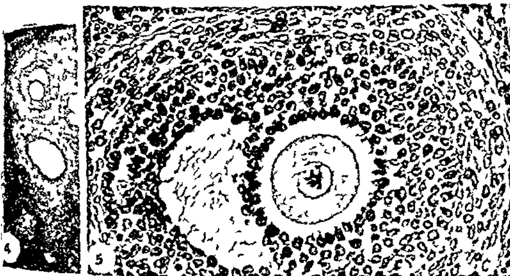
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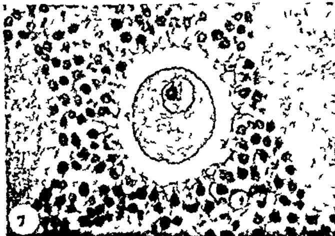
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## PLATE 1

### EXPLANATION OF FIGURES

*Normal development of oocytes and follicles*

2 Low power survey of primordial and primary follicles at periphery of ovary GP 55 — in heat × 56

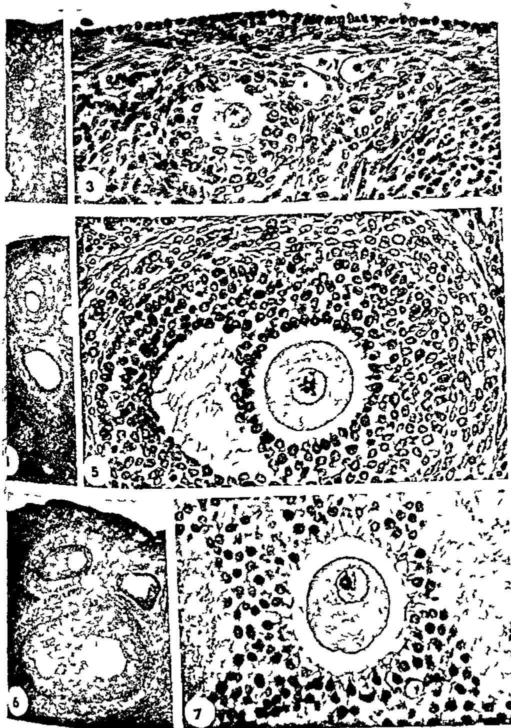
3 High power of primordial oocytes with contracted nuclei a small primary follicle and a large primary follicle with granulosa cell mitoses GP 34 Second day × 350

4 Low power of a fourth day follicle GP 38 × 56

5 High power of figure 4 to show developing theca layer and many mitotic figures in granulosa wall and cumulus Nucleus of oocyte is centrally located Projections from cells of the cumulus can be seen traversing the zona GP 38 × 350

6 Low power of an eighth day follicle to show the wide granulosa layer made up of cells loosely adherent to each other and the web like cumulus attached at several points to granulosal wall GP 36 × 56

7 High power of oocyte in figure 6 to show eccentric nucleus with vacuolated nucleolus GP 36 × 350


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## PLATE 2

### EXPLANATION OF FIGURES

*Normal development of oocytes and follicles*

8 High power of figure 9 to show oocyte of twelfth day of cycle A corona radiata is developing Extracted lipid droplets can be seen in the cytoplasm of the oocyte G P 35 × 350

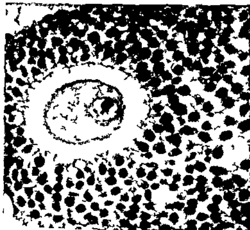
9 Low power of twelfth day follicle to show compact granulosa and enlarging cavity G P 35 × 56

10 High power of figure 11 to show just before the onset of heat nuclear changes in the oocyte with the appearance of a network of fibrils and a dense basophilic mass G P 46 × 350

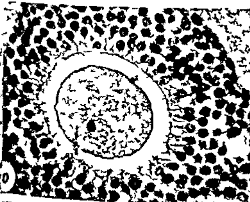
11 Low power of a sixteenth day follicle before the beginning of heat The granulosa wall is compact the cavity is enlarged and the theca is not hypertrophied Note lakes of fluid at base of cumulus G P 46 × 56

12 High power of oocyte in figure 13 from animal in heat The first maturation spindle has formed and an anaphase is seen in a tangential cut The adjacent section contained the rest of the spindle and the chromosomes of the opposite pole The cells of the corona radiata are elongated their nuclei appear pycnotic G P 62 × 350

13 Low power of follicle from an animal in heat Note dispersal of cells of the cumulus to form a loose coagulum G P 62 × 56

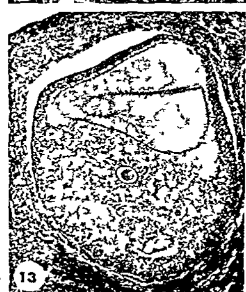



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## PLATE 3

### EXPLANATION OF FIGURES

*Adenosine monophosphatase in normally developing oocytes and follicles*

14 The flat follicular cells around these two primordial oocytes and the surrounding cortical cells show AMPase activity Note lack of activity in the oocyte GP 41 fourth day × 1 400

15 A portion of a multilayered primary follicle (right) and the contained oocyte (left) AMPase activity is seen around each granulosa cell and in a dense band formed by their projections at the periphery of the developing zona pellucida. GP 41 fourth day × 1 400

16 Low power view of a small follicle of the fourth day of the estrous cycle The dense tunica albuginea and superficial cortical layer (top) and the granulosa cells are strongly positive for AMPase activity Note atretic follicle with degenerating oocyte at lower left GP 41 × 140

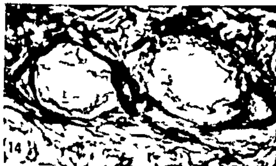
17 A high power of portion of the oocyte zona pellucida and cumulus seen in figure 16 Note AMPase activity in periphery of granulosa cells (right) in dense band of their projections at periphery of zona (center) and in their projections that traverse zona to terminate at oocyte surface GP 41 × 1 400

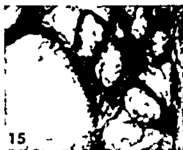
18 A portion of an oocyte (lower left) from a follicle from the eighth day of the estrous cycle The granulosa cells and their projections that cross the zona are reactive for AMPase The dense band of projections at the periphery of the zona is no longer present GP 74 × 1 400

19 A low power of a follicle developing on the twelfth day of the cycle AMPase activity is still present in the granulosal cells but has diminished since the fourth day GP 60 × 90

20 A high power of part of an oocyte (left) from a follicle just prior to the onset of heat (seventeenth day) The granulosa projections are still positive for AMPase GP 47 × 1 400

21 A high power of part of an oocyte (left) from a follicle during heat The granulosa projections though reactive for AMPase in the corona radiata are no longer seen in the zona pellucida GP 77 × 1 400


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### EXPLANATION OF FIGURES

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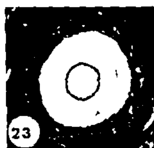
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## PLATE 4

### EXPLANATION OF FIGURES

*Nucleoside polyphosphatase in normally developing oocytes and follicles*

22 With ATP as a substrate oocytes in primary follicles have a band of activity at their periphery and usually contain reactive granules in their cytoplasm The ovarian stroma is strongly reactive the tunica albuginea weakly positive and the serosal epithelium of the ovary is negative G P 28 Fourth day × 140

23 With ATP as a substrate this oocyte has a reactive rim Compare with figure 4 G P 46 Sixteenth day × 140

24 Serial section to figure 23 With ADP as a substrate there is usually no reactivity around the oocyte and stromal activity is slightly less G P 46 × 140

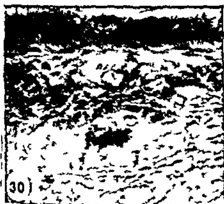
25 Reactivity with either ATP or ADP as substrates is seen in the ovarian stroma and the supporting cells of the theca interna Slight activity with ATP is seen at the periphery of the oocyte in this follicle from the fourth day of the cycle Precipitate was frequently seen over the cavity of small follicles and was interpreted to be artifactitious G P 41 × 140

26 A high power of an oocyte (left) from a small follicle of the fourth day Reactivity with ATP as a substrate is seen at the periphery of the oocyte possibly associated with ovular microvilli and in granules in the cytoplasm of the oocyte The zona pellucida (right center) and the granulosa cells (right) are negative G P 8 × 1 400

27 An oocyte and cumulus in a follicle from the twelfth day of the cycle With ATP as a substrate the oocyte has lost its peripheral rim of activity See figures 8 and 29 for serial sections through this oocyte G P 60 × 140

28 A higher power of oocyte in figure 27 With ATP as a substrate a few coarse granules of activity are seen in the cytoplasm G P 60 × 560

29 A serial section to that in figure 28 With ADP as a substrate somewhat smaller granules are seen in the oocyte The single granules seen in the cells of the cumulus are not enzymatically produced since the water blank controls contain similar granules G P 60 × 560


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## PLATE 5

EXPLANATION OF FIGURES

*Acid phosphatase in developing oocytes*

30 Acid phosphatase activity is seen in granules in the oocyte of this primary follicle G P 28 Fourth day × 350

31 Acid phosphatase water blank control in a serial section to that in figure 30 The granules seen in the serosal epithelium (top) are not enzymatically produced and may represent an inorganic phosphate reaction associated with a lipid compound G P 28 × 350

32 A small follicle of the fourth day to show acid phosphatase activity in the contained oocyte and negative granulosa An interstitial mass at left of oocyte shows faint activity G P 84 × 140

33 A higher power of the oocyte seen in figure 32 Discrete granules of activity are seen throughout the oocyte cytoplasm A faint dusting of activity is seen in the cumulus G P 84 × 560

34 A high power of a matured follicle wall to show the acid phosphatase positive macrophages that during heat invade the granulosa (right) from the theca (left) This same follicle is seen in figure 41 (Sudan black) and at the left in figure 61 (alkaline phosphatase) G P 77 × 350

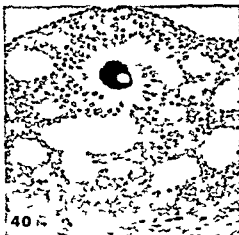
35 An oocyte from a follicle of the twelfth day showing that the acid phosphatase positive granules are clearly unassociated with the lipid droplets G P 60 × 560


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## PLATE 6

### EXPLANATION OF FIGURES

*Alkaline phosphatase and lipid in developing follicles*

36 The primordial follicles seen in the superficial cortex (top) are negative The developing theca interna of the primary follicle (bottom) and the cells of the interstitial mass are reactive for alkaline phosphatase G P 77 — in heat × 280

37 The oocyte and granulosa layer are negative for alkaline phosphatase The theca interna is strongly positive G P 77 — in heat × 280

38 The sudanophilia in this oocyte in a primary follicle is diffuse and probably represents mitochondrial staining G P 74 Eighth day × 280

39 Sudanophilic droplets appear in oocytes in developing follicles of the fourth day The interstitial mass surrounding this follicle shows dense sudanophilia G P 84 × 140

40 This mature pre estrous follicle of the seventeenth day shows sudanophilia in the oocyte and in the macrophages of the theca interna Its granulosal wall at higher magnification showed fine sudanophilia G P 47 × 140

41 During heat the granulosal cells rapidly become sudanophilic This follicle is also seen in figure 34 (acid phosphatase) and at the left in figure 61 (alkaline phosphatase) G P 77 × 350

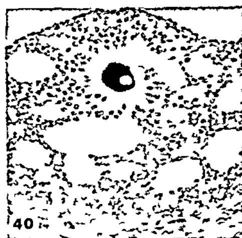
42 A high power of the oocyte seen in figure 40 showing sudanophilic droplets throughout cytoplasm G P 47 × 560


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## PLATE 7

### EXPLANATION OF FIGURES

*Atretic follicles and the development of interstitial masses*

43 The granulosa layer in this primary follicle is disorganized The oocyte has an eccentrically located nucleus G P 47 Seventeenth day × 140

44 A small follicle whose cumulus is thin Several mitoses are seen in the granulosa layer Note extreme eccentricity of the nucleus in this oocyte G P 40 Twelfth day × 140

45 The granulosa layer in this small follicle is sloughing into the cavity G P 62 — in heat × 140

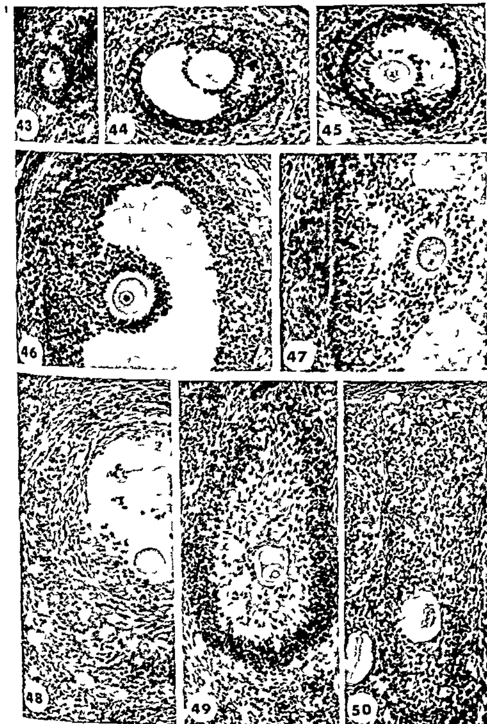
46 This is an early atretic follicle owing to the granulosal slough and thecal hypertrophy Note mitoses in granulosa in spite of atresia The oocyte appears histologically normal G P 35 Twelfth day × 140

47 The granulosal layer has degenerated there is a maturation spindle in the oocyte the theca interna layer is wide and contains many mitoses G P 62 — in heat × 140

48 This follicle with advanced atresia shows complete shedding of the granulosa layer beginning organization at the periphery of the cavity and a wide thecal layer The oocyte is degenerating G P 65 Twelfth day × 140

49 This follicle has a more advanced stage of organization of the former cavity an oocyte that has undergone fragmentation or cleavage and a thecal layer now forming an interstitial mass G P 64 — in heat × 140

50 An interstitial mass showing remnants of an oocyte within its central cavity G P 38 Fourth day × 140


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## PLATE 8

### EXPLANATION OF FIGURES

*Atretic follicles with invasion of granulosa by vascularized connective tissue from the theca*

51 Delicate strands from the theca invade the granulosa and cumulus in this eighth day follicle G P 36 × 140

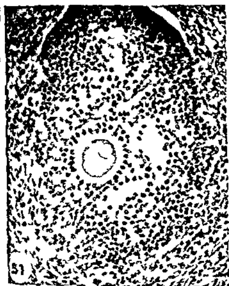
52 Nucleoside polyphosphatase activity is present in the invading cells G P 74 Eighth day × 140

53 Two similar follicles of the eighth day one of which shows evidence of early atresia in the nucleoside polyphosphatase positive invasive thecal elements (right) Follicle on left is normal Compare with a nearby section of same two follicles (alkaline phosphatase) in figure 55 G P 74 × 56

54 A thecal invasion with small blood vessels near the base of a cumulus There are a few sloughed granulosal cells Note apparently normal oocyte G P 40 Twelfth day × 140

55 Alkaline phosphatase reaction on same two follicles as in figure 53 Note the absence of this enzyme in the cumulus on the right This indicates that the invasive strands are not the alkaline phosphatase positive thecal cells G P 74 × 56

56 A follicle with advanced atresia showing a stalk of cells continuous with the theca at base of former cumulus G P 65 Twelfth day × 140


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## PLATE 9

### EXPLANATION OF FIGURES

*Atretic follicles — alkaline and neutral phosphatases*

57 This follicle of the thirteenth day shows minimal atresia. The basal layers of the granulosa are *nucleoside* polyphosphatase positive. See figure 58 for higher power of follicle wall and figures 64 and 72 for histochemical evidence of abnormality in its contained oocyte.

58 High power of wall of follicle shown in figure 57. The granulosa layer is beginning to slough *into* the cavity distally and its basal layers are nucleoside polyphosphatase positive. C P 51. × 560.

59 With ATP as a *substrate* the oocytes in the non-developing small follicles found throughout the cycle frequently maintain a peripheral rim of activity. The large follicle at lower *left* shows advanced sloughing and a basal granulosal layer with nucleoside *polyphosphatase* activity. G P 14. Twelfth day. × 140.

60 Sodium *glycerophosphate* at pH 7.2 was used as a control for the specificity of the nucleoside polyphosphatase reactions. It gave a moderate reaction in the thecal layer *only in* locations similar to that seen with alkaline phosphatase. The granules in the granulosa are not enzymatically produced since they are present also in the water blank control. C P 51. Thirteenth day. × 140.

61 The follicle at left is *matured* and about to ovulate. Its theca interna is not prominent and shows only moderate alkaline phosphatase activity as compared with the two *non-ovulating* follicles (at the right) with hypertrophied theca. G P 77 — in heat. × 88.

62 This shows a variety of *stages* of interstitial masses. Alkaline phosphatase remains prominent in the thecal layer as it becomes transformed into an interstitial mass. G P 74. Eighth day. × 88.


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## PLATE 10

### EXPLANATION OF FIGURES

*Atretic oocytes — Adenosine monophosphatase and lipid*

63 This follicle shows beginning atresia in the sloughing granulosal layer The sloughed granulosal cells lose their AMPase activity See figure 65 for details of oocyte G P 44 Twelfth day × 140

64 This oocyte has abnormally large lipid droplets in its cytoplasm This section is serial to those seen in figures 72 and 73 The follicle wall showed minimal atresia (see figures 57 and 58) G P 54 Thirteenth day × 560

65 A higher power of the oocyte seen in the atretic follicle in figure 63 The AMPase reactive terminals of the granulosal projections are enlarged at the oocyte surface G P 44 Twelfth day × 560

66 The oocyte in this atretic follicle shows clumped or coalesced lipid droplets The shedding granulosa remains negative for sudanophilia G P 44 Twelfth day × 140

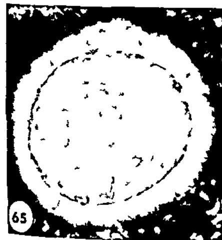
67 This oocyte from a follicle that showed only moderate sloughing of the granulosa layer nevertheless contains a dense band of AMPase activity at the periphery of the oocyte This is interpreted to result from enlargement or possibly fusion of the terminals of the granulosa projections G P 84 Fourth day × 140

68 The surviving granulosal cells still retain their AMPase activity in this follicle with advanced atresia and a fragmented or segmenting oocyte G P 84 Fourth day × 140

69 This atretic follicle is developing into an interstitial mass Its oocyte has large clumps of sudanophilia The macrophages in the organizing cavity contain lipid The theca cells only gradually develop sudanophilia G P 28 Fourth day × 140


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## PLATE 11

### EXPLANATION OF FIGURES

*Acid phosphatase in atretic oocytes and follicles*

70 A small follicle with disorganized granulosa cells contains an oocyte with clumped acid phosphatase activity G P 41 Fourth day × 140

71 A larger follicle with minimal sloughing of the granulosa layer contains an oocyte with clumped acid phosphatase activity G P 74 Eighth day × 1 0

72 This oocyte with clumped acid phosphatase activity was found in the follicle with minimal atresia shown in figures 57 and 58 Serial sections of the oocyte are seen in figure 64 (sudanophilia) and figure 73 (acid phosphatase control) G P 54 Thirteenth day × 560

73 Acid phosphatase water blank control through a serial section of the oocyte in figure 72 No precipitate is seen G P 54 Thirteenth day × 560

74 With moderate to advanced atresia the oocyte shows reduced or no acid phosphatase activity The shedding granulosal layer remains negative Macrophages in the thecal layer (right) contain reactive granules G P 44 Twelfth day × 560

75 With advanced atresia the oocyte is negative for acid phosphatase activity The macrophages in the organizing cavity are positive and the thecal layer becomes faintly positive as it is transformed to an interstitial mass G P 28 Fourth day × 140

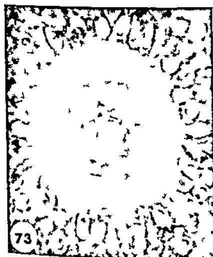
76 Occasionally degenerating oocytes contain a substance that blackens in both this acid phosphatase preparation and in its water blank control (fig 77) This precipitate is characteristic of the presence of inorganic phosphates G P 28 Fourth day × 140

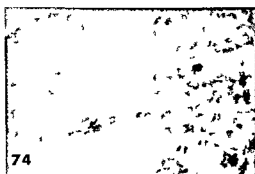
77 Acid phosphatase water blank control on a section serial to that in figure 76 to show that the black precipitate in the degenerating oocyte is not enzymatically produced G P 28 Fourth day × 140

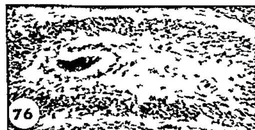

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## PLATE 12

### EXPLANATION OF FIGURES

*Atretic oocytes with abnormal nuclear changes*

78 This follicle shows little histologic evidence of atresia yet the nucleus of its oocyte is in the first maturation spindle This nuclear change is seen in most oocytes about the time of estrus G P 34 Second day × 140

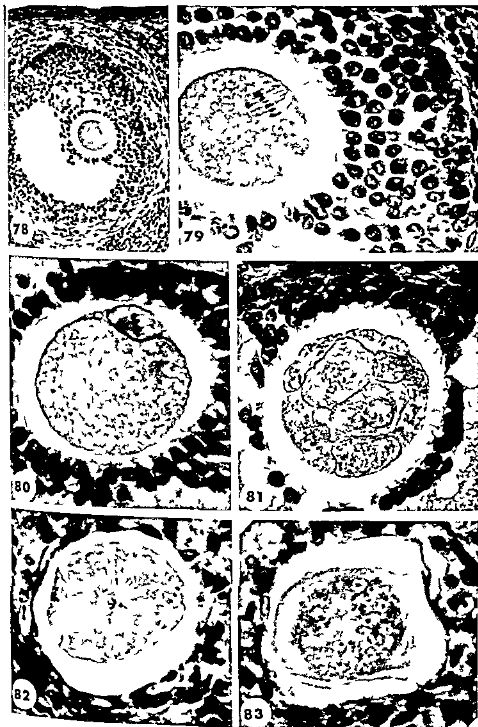
79 A higher power of figure 78 to show maturation spindle There are definite mitotic figures in the granulosal layer (lower right) G P 34 second day × 560

80 This oocyte shows a spindle in the polar body and another section contained the second maturation spindle It was contained in an atretic follicle with moderate sloughing of the granulosal layer Note mitosis in cumulus G P 60 twelfth day × 560

81 This oocyte appears to have undergone several cleavages to form a morula like structure The atretic follicle which contained it had a very thin granulosa layer Note poorly formed cumulus No granulosa slough was apparent G P 34 Second day × 560

82 This oocyte is degenerating in an atretic follicle that is becoming organized to form an interstitial mass A polar body is seen at left The large astral ray with associated basophilic clumps appears to be derived from the oocyte nucleus G P 47 seventeenth day × 560

83 This degenerating oocyte in an organizing follicle contains several micronuclei without any evidence of cytoplasmic cleavage G P 40 Twelfth day × 560


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# Differentiation of Mouse Thymus Cultured in Diffusion Chambers

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ABSTRACT Considerable evidence has been brought forward in recent years to show that thymus growing in intraperitoneally implanted diffusion chambers will partially restore immunological competence to mice thymectomized at birth (Osoba and Miller 63) Levey Trainen and Law 63 Law Trainen Levey and Barth 64 Law Dunn Trainen and Levey 64 Osoba 65) Since it was suggested that the thymus cells secrete a factor that mediates the restoration it was of interest to examine the thymic cells that grew or survived in the diffusion chamber Whole thymuses from new born mice were grown in thymectomized or intact syngeneic or allogeneic hosts of various ages Whole mount preparations were made of the thymus tissue after it had grown for from 9–86 days Thymic lymphocytes granulocytes macrophages and fibroblasts migrated rapidly from the explant, the latter forming a sheet that covered the entire chamber Viable lymphocytes were present in chambers removed from the host after 86 days The striking feature of the cultures was the differentiation of the epithelium into sheets clusters and cords of cells reminiscent of glandular epithelium Whorls of cells resembling Hassall s bodies were occasionally seen These epithelial cells were distinguished by the presence of large lipid filled vacuoles and neutral mucopolysaccharide in the cytoplasm It is suggested that the epithelial cells were engaged in secretory activity

Considerable evidence has been brought forward in recent years to show that embryonic or neonatal thymus growing in intraperitoneally implanted diffusion chambers will partially restore immunological competence to mice thymectomized at birth (Osoba and Miller 63 64 Levey Trainen and Law 63 Law Trainen Levey and Barth 64 Law Dunn Trainen and Levey 64 Osoba 65) These and other experiments (Metcalf 56 60) have suggested that the thymus cells secrete a "humoral substance" that mediates the restoration and for this reason it has been of interest to examine the type of cells that grow or survive in the diffusion chamber Previous descriptions of thymic tissue grown in diffusion chambers which have relied upon the examination of sections of paraffin-embedded material have shown that epithelial reticular" cells and possibly lymphocytes (Levey Trainen and Law '63 Osoba 65) may survive for periods as long as 18 weeks however these preparations provide limited information as to the survival and growth pattern of the thymic tissue as a whole The descriptions that follow are based upon "whole mounts" of the thymic tissue recovered from intraperitoneally implanted diffusion chambers These show that the epithelial cells grow as sheets or clusters or as a network of cords and that thymic lymphocytes while steadily decreasing in numbers may continue to survive for as long as 12 weeks

## MATERIAL AND METHODS

Thymuses and in one experiment spleens were removed as nearly intact as possible from new born donors that were anesthetized with ether or by low temperature They were blotted free of blood on filter paper soaked in chilled phosphate buffered saline pH 7 2 and dissected free of extraneous tissue The tissues were kept on ice in a Petri dish containing a saline moistened filter paper until they were put into the chambers The center filter of each chamber was moistened with saline before the tissues were enclosed by sealing the top filter Immediately after sealing each chamber was inserted into the peritoneal cavity of the host mouse and the

abdominal muscle and skin were closed separately with silk sutures Sterile technique was used throughout

Double diffusion chambers of a design previously described (Shelton and Rice, 58) were used They consisted of two lucite rings (20 mm o d , 10 mm i d , for adult hosts 12 mm o d 6 mm i d for three week hosts) between which a Millipore filter [1] (type HA pore size 0 45 μ ± 0 02 μ) was cemented On the outside of the rings were cemented Schleicher and Schuell Bac T Flex B12 filters [2] (average pore diam 0 15 μ) The chambers were fabricated except for the top filter and sterilized by dry heat at 80°C for 48 hours

A total of 53 chambers containing thymus and eight chambers containing spleen are included in this study The following combinations of donor → host were used BalbC → BalbC, BalbC → A $C_3H \rightarrow (C_{57}Bl \times C_3H)F_1$, $C_{57}Bl \rightarrow (C_{57}Bl \times C_3H)_{F1}$ and DBA/2 → DBA/2 In the thymus series 12 of the host mice were three weeks of age and had been thymectomized at birth 33 were three months of age and were intact The spleens were put into three week old thymectomized hosts

When the chambers were removed from the animals, the top filter was removed and the accumulated fluid was aspirated from around the tissue with a pipette and rubber bulb Small droplets of this fluid were placed on slides for examination with the phase microscope The bottom filter of the chamber was removed the fluid discarded and the center filter on which the thymus was growing was fixed in one of the following fixatives

(1) 3% potassium dichromate (pH 6) to which 10% formalin had just been added, (Orth s fluid)

(2) 95% ethyl alcohol to which 10% of formalin had just been added (alcohol formalin)

(3) 10% formalin 2% calcium acetate 0 5% cetylpyridinium chloride in water (CPC formalin)

With the exception of one group of eight thymus chambers the tissues were fixed processed and mounted on slides as whole mounts (Shelton and Rice 58) Tissue from the group of eight thymus chambers was sectioned frontally" after paraffin embedding Material fixed in Orth s fluid was stained with Giemsa s stain Lillie s allochrome connective tissue stain and iron hematoxylin tissues fixed in alcohol formalin were stained in azure-eosin hematoxylin, tissues fixed in CPC formalin were stained with oil red O for lipid (Lillie '54) and with alcian blue PAS (after diastase digestion) for mucopolysaccharide (Spicer, 60)

## RESULTS

Neither the genetics of the donor → host combination nor the age nor the presence or absence of the thymus in the host affected the growth pattern or the survival of the thymus in the diffusion chambers The explanation of the gland to the diffusion chamber resulted in concommitant death and proliferation of cells and, as is common with all cultures of organs and tissues there was considerable variability in the growth pattern and cell survival from chamber to chamber Complete necrosis of the tissue was never observed but necrosis was always present in the central portion of the explant Dying thymic lymphocytes were quite clearly recognized but it was difficult to ascertain to what extent the epithelial stroma of the glands was involved in the necrotic process Lymphocytes present in profusion in some chambers were sparse in others. Similar variability was observed in the proliferation of sheets and cords of epithelium but in only five of the 45 whole mount preparations examined were epithelial cells missing entirely In these five preparations only fibroblasts proliferated and it is worth remarking that very little evidence of necrotic cells could be detected a fact which attested to the remarkable activity of the macrophages in the chambers Table 1 shows the number of whole mounts of thymus tissue examined at various time intervals

The tissue of the freshly explanted thymus was too compact to be examined as a whole mount but after nine days of growth in the diffusion chamber it had loosened up and spread out so that its structure could be discerned During these nine days fibroblasts had migrated out from the gland to form two thin sheets which

[1] Millipore Filter Corp Bedford Massachusetts

[2] Carl Schleicher and Schuell Co Keene New Hampshire

TABLE 1

| Chambers | 9–15 day | 26–40 days | 55–86 days |
|---|---|---|---|
| otal with epithelial proliferation | 17 | 19 | 4 |
| otal without epithelial proliferation | — | 4 | 1 |

xtended on the roof and the floor to the edges of the chambers There were many free macrophages and thymic lym hocytes present in the fluid of the cham er and wandering between and on the urface of the sheets of fibroblasts The pithelial portion of the gland remained ocalized where it had been initially placed nd reorganization and growth of the epi helial cells had begun In favorable prep- rations the outline of each of the two hymic lobes could be seen as a pattern of lusters or interconnected cords of epithe lal cells completely surrounded by fibro- la‚s (fig 1) These clusters and cords f cells were only one or two cells thick although they could be many cells wide (fig 2) During the first two weeks of growth thymic lymphocytes were invari ably present floating free in the fluid of the chamber or scattered in clusters throughout the solid tissue on the filter Clusters of granulocytes and occasional foci of myelopoiesis were seen in several of the chambers and mast cells were ubiquitous

Later as the tissue of the thymus spread out more extensive and elaborate prolifer ation of the clusters and cords of cells be came evident In some chambers sheets of epithelium were more prominent than cords or clusters while in others both sheets and cords were present After four weeks epithelial cells were still actively proliferating (figs 3 4 5) and many thy mocytes were present in the chamber fluid (figs 7 8 9) and intercellular spaces of the tissue Whorls of cells strongly re sembling Hassall s bodies were now pres ent at intervals along the epithelial cords (fig 6) By 55 days thick cords of cells could still be seen (figs 10 11 12) but many now had become more irregular in outline and their thin walls enclosed an empty or necrotic lumen Clusters of thy mocytes were still present (fig 12) In chambers opened between 70 and 86 days sheets and cords of cells still persisted and clusters of viable lymphocytes were ob- served (figs 13 14 15)

In addition to a characteristic pattern of growth a feature distinguishing the epi thelial cells was the presence of large vacuoles in the cytoplasm (figs 2 11)

When explants that had grown in the chambers for from 9 to 28 days were stained with oil red O the vacuoles were found to contain lipid (figs 18 19) When other explants were digested with diastase to remove glycogen and then were stained with Alcian Blue PAS the cytoplasm of the epithelial cells were PAS positive in dicating the presence of neutral muco- polysaccharide (fig 20) (Spicer 60) The vacuoles in the cytoplasm did not stain with PAS but accumulations of PAS positive material were found in what appeared to be a lumen in some of the cords of cells (fig 21) Accumulation of lipid and PAS positive material in macrophages varied greatly and appeared to be related to the state of the phagocytic activity of the in dividual cells Surprisingly lipid droplets were also found in some of the thymic lymphocytes and presumably the vac uoles seen in the lymphocytes after 86 days in culture (figs 14 15) were also due to the presence of lipid The fibro- blasts frequently contained very fine dust like lipid droplets and were very faintly PAS positive

The serial sections of the eight paraffin embedded tissues recovered from chambers after four weeks of growth would have been exceedingly difficult to interpret had it not been for the prior experience with the whole mounted tissues The epithelial sheets and tubules were so thin that they were not easy to follow through the 5 μ sections and it would have been almost impossible to recognize that the clusters of epithelial cells scattered through the fibroblastic matrix were sections through a network of cords However it was easy to see in the sections that the center of the explant was necrotic and it appeared that the proliferation of the epithelium came mainly from cells in the cortex of the gland It thus appeared probable that the central necrosis of the explant involved all cell types

*New born spleen* The eight chambers containing new born spleen were opened after four weeks The growth consisted of flat sheets of reticular cells (fig 16) with clusters of macrophages and polymorpho nuclear leucocytes In three of the cham bers, extensive myelopoiesis was observed (fig 17) If lymphocytes were present they could not be distinguished

## DISCUSSION

In the past experiments with the *in vitro* cultivation of the thymus were largely concerned with the problem of the origin of the thymic lymphocytes, but some in vestigators (Emmart 36, Popoff 26 Mur ray 37) did describe the characteristics of the epithelial outgrowth of their cultures Popoff ( 26) a student of Maximow be lieved that the thymic lymphocytes origi nated from the mesenchyme and migrated into the epithelial rudiment of the gland and he thus considered the question of the origin of the thymic lymphocyte to be a closed issue He concentrated his efforts therefore, upon the examination of the characteristics of the epithelium of 100 ex plants from new born half grown and adult rabbits It is worth noting from the point of view of technique that these cul tures were prepared from fragments of minced tissue since the explanation of whole organs into a static culture environ ment usually met with no success because of insufficient gas and nutrient exchange in thick explants and in spite of this he always observed necrosis in his cultures He found as we have that the epithelium remained relatively stationary while lym phocytes histiocytes and polymorphonu clear leucocytes migrated rapidly from the explants and that the fibroblasts formed a sheet that eventually enveloped the epi thelium In his cultures the epithelium con tracted to form islands and he noted that clear vacuoles became prominent in and between the cells of the epithelial foci that they could attain quite a large size and were probably to be looked upon as a manifestation of a kind of secretory ac tivity In his conclusions Popoff states unequivocally that the epithelial cells were engaged in secretory activity Twenty-one years later in the same laboratory, Murray ( 47) succeeded in obtaining pure cultures of rabbit thymus epithelium which he said grew in tongues sheets and cords The illustrations of his cultured cells are re markably similar to figures 4 and 5 of the present paper He concluded that the stroma of the gland was entirely epithelial and that there was no difference between the medulla and the cortex of the gland so far as the capacity for epithelial pro liferation was concerned

The evidence that the cells of thymus glands grown in diffusion chambers se crete a diffusable substance that influences the immunological reactivity of the hosts has raised the question of the cellular origin of the substances and provided the impetus for examining the morphology of such cultures It was perhaps naive to expect successful cultivation of the ex plants of whole thymic lobes Necrosis of the cells in the center of the explant promptly occurred but at the same time there was growth and reorganization of the epithelial cells and a remarkably rapid proliferation and migration of the fibro blasts and wandering" cells of the gland This concomitant death migration an growth produced a sheet of cells th enough to permit the penetration of ga and nutrients so that successful cult were established

The striking feature of the diffusion chamber cultures was the epithelial differ entiation a feature for which the morpho logical appearance of the gland in the adul animal provides little evidence althoug the embryonic anlage of the thymus i clearly epithelial These cells proliferate and rearranged themselves into structure very reminiscent of glandular epithelium [Murray ( 47) could not distinguish be tween the epithelial ougrowth from cu tures of either gall bladder intestine c thymus so similar was their morphology The question to be answered is are the thymic epithelial cells engaged in secretor activity and are they secreting a materi that influences the immunological com petence of the host? In agreement wit the conclusions of others (Osoba an Miller 64 Metcalf 60 Popoff '26 circumstantial evidence implicates the ep thelial cell as being involved in such ac tivity if indeed it exists The histochem ical evidence shows that while lipid ar

-AS positive material was present in macrophages lymphocytes and fibroblasts these materials were more consistently present in the epithelial cells and served clearly to distinguish them from all other cells in the chambers In addition it has been shown (Osoba 65) that diffusion chamber cultures of new born spleen are ineffective in restoring the immunological competence of the host and it was shown in the present experiments that spleen cultures are devoid of epithelial cells Finally the lymphocytes are probably not involved in the restoration of immunological competence since although some were still present after 86 days in the chamber their number steadily decreased from the time of explantation and they could be considered to be a dying population

In experiments where the immunological capacity of the host has been restored considerable animal to animal variation has been observed (Osoba and Miller 63 64 Osoba 65 Law Trainen Levey and Barth 64) Such variation may have been due to variation in the survival of the thymic epithelium Further experiments designed to correlate the survival of specific cell types in the chamber with the immunological reactivation of the hosts should provide more insight into the function of the thymus in diffusion chamber cultures

ACKNOWLEDGMENTS

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## PLATE 1

### EXPLANATION OF FIGURES

1 Low power view of BALB/c thymus explant after nine days of culture in an intact adult BALB/c host The fenestrated pattern formed by the growth of the thymus epithelium clearly delineates the two lobes of the thymus Arrow points to a contracted sheet of fibroblasts that had pulled off the top filter of the chamber when it was removed Allochrome stain 23 ×

2 Enlargement of a portion of the thymus shown in figure 1 Broad cords of epithelial cells are surrounded by a matrix of fibroblasts Living and dead thymocytes are dispersed singly or in clusters in the fibroblastic matrix The preparation is too dense to permit good resolution of cellular detail but the epithelial cells can be seen to be filled with large vacuoles (compare with fig 11) These vacuoles correspond to the large lipid deposits seen in appropriately stained material 500 ×




2

## PLATE 2

### EXPLANATION OF FIGURES

3 Twenty-eight day-old explant of $C_{57}$ Black thymus placed into three week old thymectomized $(Bl \times C_3H)_{F1}$ host Epithelial cords and tubules have proliferated extensively into the sheet of fibroblasts Azure eosin 100 ×

4 Detail of epithelial cord of cells seen in figure 3 Note lymphocytes still present in connective tissue next to epithelial cord 500 ×

5 Detail of epithelium seen in figure 3 Mitoses can be seen in this proliferating cord of cells 500 ×

6 Hassall s bodies are formed by some of the epithelial cells Same culture as figure 3


3


4


5


6

## PLATE 2

### EXPLANATION OF FIGURES

3 Twenty-eight day-old explant of $C_{57}$ Black thymus placed into three-week-old thymectomized $(BI \times C_3H)_{F1}$ host Epithelial cords and tubules have proliferated extensively into the sheet of fibroblasts Azure-eosin 100 ×

4 Detail of epithelial cord of cells seen in figure 3 Note lymphocytes still present in connective tissue next to epithelial cord 500 ×

5 Detail of epithelium seen in figure 3 Mitoses can be seen in this proliferating cord of cells 500 ×

6 Hassall s bodies are formed by some of the epithelial cells Same culture as figure 3





## PLATE 3

### EXPLANATION OF FIGURES

Representative phase optical photographs of the cell population in the fluid of a culture of new born BALB/c thymus grown for 26 days in an intact adult strain A host

7 Thymic lymphocytes two of which display typical hand mirror form of locomotion 2 000 ×

8 Two thymic lymphocytes and a macrophage in metaphase 2 000 ×

9 Group of thymic lymphocytes and macrophages The large vacuol in the macrophage at the left probably corresponds to the PAS posi tive material seen in stained preparations 1 000 ×

10






## PLATE 4

### EXPLANATION OF FIGURES

10 Fifty five days after explantation of BALB/c thymus into intact adult BALB/c host the pattern of epithelial cord like structures can clearly be distinguished from the surrounding connective tissue Giemsa stain 80 ×

11 A cord of epithelial cells thrusts into the sheet of fibroblasts covering the floor of the chamber Many of the cells contain large vacuoles which probably represent lipid inclusions Detail of figure 10 500 ×

12 In this epithelial cord there is very little evidence of lipid inclusions although some of the cells appear to be degenerating Thymic lymphocytes which are still viable at this time can be seen scattered or in clusters in the fibroblastic matrix of the culture Detail of figure 10 500 ×


11



## PLATE 5

### EXPLANATION OF FIGURES

13 Eighty six days after explantation of BALB/c thymus into an intact adult BALB/c host pale staining tubules can still be seen Only the outer cells of the tubules are still viable at this time Lymphocytes can be seen as pepper like dots Giemsa stain 80 ×

14–15 Detail of clusters of thymic lymphocytes present in 86-day-old thymus culture shown in figure 13 Many of the lymphocytes contain vacuoles which are presumed to denote the presence of lipid 1 000 ×

16 Spleen from new born $C_{57}$ black donor grown in diffusion chamber placed into three week old thymectomized $(C_3H \times C_{57}Bl)_{F1}$ host The reticular cells form a criss-cross pattern in some areas of the culture Twenty nine days Azure eosin 500 ×

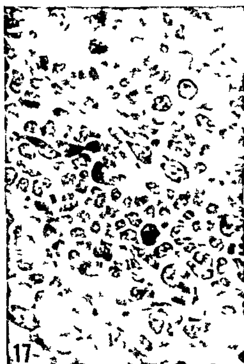
17 Extensive myelopoiesis was present in several of the spleen cultures Twenty nine days 500 ×


13


14




16


17

## PLATE 6

### EXPLANATION OF FIGURES

18 DBA/2 thymus stained for lipid after 11 days of growth in an intact DBA/2 host The epithelial cells forming long strands in the center of the explant can be distinguished by the accumulation of lipid droplets in the cytoplasm The discrete dense red deposits seen in this preparation are lipid containing vacuoles in the cytoplasm of macrophages Oil red O with hematoxylin counterstain 65 ×

19 High power view of the strand of epithelial cells showing detail of lipid deposits in the cytoplasm The discrete densely stained bodies scattered through the preparation represent the deeply stained lipid deposits in the cytoplasm of macrophages Same preparation as shown in figure 18 500 ×

20 DBA/2 thymus after 25 days of growth in intact DBA/2 host The red stained material in the cytoplasm of the epithelial cells growing in closely packed cord like arrangements denotes the presence of neutral mucopolysaccharide The hypertrophied cytoplasm of two macrophages also contains PAS positive material Alcian Blue Periodic acid Schiff reaction after diastase digestion 400 ×

21 Higher power view of same preparation shown in figure 20 showing the accumulation of PAS positive material in what appears to be a lumen in a cord of epithelial cells 500 ×

# Suprarenal Gland Lymphatic Drainage [1]

ROBERT J MERKLIN
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*ABSTRACT* *The gross and microscopic anatomy of the suprarenal gland lymphatic* system was studied in post mortem specimens and specimens in which the venous system was injected with blue latex solution

Extensive subserous lymphatic networks overlie the suprarenal gland Deep to these networks are the lymphatic vessels proper of the suprarenal gland These vessels lie within the suprarenal gland capsule and communicate with the subserous lymphatic network Their primary routes of drainage however are directed medialward passing to the thoracic duct or cisterna chyli either directly or by way of regional lymph nodes A few capsular lymphatic vessels extend toward the diaphragm or the kidney The regional lymph nodes are small few in number and situated at the lower medial aspect of the gland

Microscopic examination of serially sectioned suprarenal glands revealed a complete absence of lymphatic vessels in the cortical and medullary parenchymal tissue Lymphatic vessels were found only in the capsule and in the adventitia of the central vein and its major tributaries

A close association between capsular lymphatic vessels and veins was noted and in a number of specimens capsular lymphatic vessels were traced to the inferior vena cava where they assumed a close relationship with vasa vasorum

Although studies of the lymphatic system go back several centuries the methods applied to their study have undergone little change down to the present time Parke and Michels (63) reviewed these methods and remarked that the only new procedure since the seventeenth century has been lymphangiography Even this procedure they pointed out has limitations in common with earlier methods namely getting the radiopaque material into the lymphatic vessels This requirement is a serious obstacle in lymph vessel studies since they are difficult to inject in either anagrade or retrograde fashion Parke and Michels described a technique for studying subserous lymphatic vessels that overcame this problem They applied solutions of hydrogen peroxide to post mortem specimens and found a fairly selective inflation of lymphatic vessels under this treatment In their hands a 3% solution of hydrogen peroxide was found most effective since it produced a gaseous swelling of lymphatics lasting several hours This period of time is adequate for gross study photographic recording and preparation of specimens for additional histological study I have applied the technique to a study of the suprarenal gland lymphatic system and a search for lymphaticovenous connections between the suprarenal gland and the inferior vena cava or its tributaries

## MATERIAL AND METHODS

Sixty eight adult post mortem specimens were studied by the technique of Parke and Michels Each specimen consisted of the suprarenal gland (24 left 44 right) and surrounding tissue including a portion of the diaphragm portions of the renal artery and vein and either the aorta or inferior vena cava Most specimens included both major vessels thus insuring the presence of the thoracic duct Three per cent hydrogen peroxide was applied before and during the dissection of the serous membrane and fascia covering the suprarenal gland Repeated applications of hydrogen peroxide were made as the dissections progressed

In addition selected histological material from previous studies of the suprarenal blood vessels was reexamined with the focus of attention on the lymphatic vessels This material consisted of ten adult and ten neonatal suprarenal glands in which the venous system had been in

[1] Supported by research grant HE 08441-03 of the National Institutes of Health of the United States Public Health Service

jected with blue latex before fixation, staining and sectioning In these histological sections the lymphatic channels were identified by structure and absence of injection material in their lumina Serial sections of each gland were followed in a search for tributaries from the cortex or medulla with particular care given to the area where the suprarenal vein makes its exit from the gland Sections of the inferior vena cava containing adventitial lymphatic vessels were also examined microscopically to determine their relationship to the vein

## RESULTS

Lymphatic vessels occur in great profusion under the serous lining of the abdominal cavity The gaseous swelling of lymphatic vessels produced by hydrogen peroxide solution makes them easily visible in the connective tissue and fascia and distinguishes them from accompanying veins (figs 1 2 3) They may be followed for considerable distances through the tissues as the dissection progresses and they respond to repeated application of the hydrogen peroxide solution

In the area of the suprarenal gland the subserous lymphatic channels form an extensive plexus running at several levels and in several directions within the fascia and loose connective tissue covering the suprarenal gland Deeper still are the lymphatic channels proper of the suprarenal gland These vessels are within the suprarenal gland capsule and usually number four to five on the ventral surface and two to three on the dorsal surface In figures seven and eight all the capsular lymphatic vessels seen in the dissection of 68 gross specimens are recorded in superimposed fashion The purpose of this is to show the variation in position of these vessels and the prevalence of vessels in the vicinity of the suprarenal vein Figure nine is a composite drawing intended to show a typical lymphatic drainage pattern for both glands Study of gross specimens and serial sections of the histological material mentioned under material and methods established the fact that these suprarenal capsular channels begin blindly at scattered points on the gland surface usually in close association with one of the capsular veins that form a small but important part of the suprarenal gland venous drainage system Histological study revealed the additional fact there are no tributaries from the cortical or medullary parenchyma to these capsular lymphatic channels This observation was carefully verified by comparison of the capsular veins and lymphatic vessels Since the former contained blue latex they were easily distinguished from the lymphatic vessels containing pink staining plasma While numerous venous channels were seen coming from within the gland and joining capsular veins there was not a single instance of similar lymphatic tributaries to the capsular lymphatic vessels The possibility that parenchymal lymphatic channels exist but follow a drainage route along the central vein was considered and carefully investigated Lymphatic channels were found in the connective tissue surrounding the central vein and its larger tributaries but they began blindly and had no tributaries from the parenchymal tissue In the neonatal gland the central vein and its larger tributaries are surrounded by a wide band of connective tissue and muscular tissue is absent In the adult gland prominent longitudinal muscle bundles are present in the connective tissue and the lymphatic channels are usually found external to the muscle tissue (fig 5)

The majority of capsular lymphatic channels run medialward to the thoracic duct often without the intervention of a lymph node Some channels run downward toward the kidney or superiorward toward the diaphragm More channels are found on the ventral than on the dorsal surface and on either surface their course generally parallels that of the suprarenal gland blood vessels Regional lymph nodes that is nodes specifically associated with the suprarenal gland are few in number and rather small They are usually situated medial to the lower portion of the gland

In over a dozen instances capsular lymphatic channels from the right suprarenal gland were traced directly to the surface of the inferior vena cava and appeared to terminate in this vessel (fig 6) Microscopic study was required to clarify the

lationship The lymphatic channels were und to take a position alongside vasa asorum of the inferior vena cava The ninal walls of these two structures were en to come in contact (fig 6) and the mphatic channel abruptly ended just be re the vasa vasorum emptied into the ena cava In summary there was no apparent lymphaticovenous connections in ny of the specimens examined in this tudy

## DISCUSSION

Although there have been earlier studies f visceral lymphatics the first compre hensive report on adrenal gland lym phatics was made by Kumita (09a b) He repeated studies by Stilling (98) on bo ine adrenal lymphatic vessels and carried out additional studies on canine and new born human adrenal glands Kumita s re sults substantially the same as Stilling s were that the adrenal gland of lower forms and of man were well supplied with lym phatic vessels

According to Kumita these vessels are disposed throughout the capsule cortex and medulla and drain into a lymphatic meshwork surrounding the central vein This meshwork in turn empties into two lymphatic trunks accompanying the supra renal vein and terminating in regional lymph nodes Not only did Kumita de scribe intercellular lymphatic channels but he reported and included figures of what he believed to be intracellular lymph channels

Aside from a number of abbreviated reports 50 years elapsed before another comprehensive study was made on this subject In 1959 the Russian Sapin published a study of the lymphatic system in 120 adult human specimens He reported essentially the same distribution of lym phatic vessels in the cortex and medulla as Kumita reported except for the drain age pattern of the cortex Sapin reported a centrifugal drainage of the cortex into capsular lymphatic vessels rather than a drainage inward into the medullary vessels

My results differ substantially from those of Kumita and Sapin In the present investigation suprarenal gland lymphatic vessels were found only in the capsule of the gland and in the adventitia of the cen tral vein and its largest tributaries It would seem that Kumita and Sapin mis took the medullary venous sinuses and the small veins passing out through the cortex into the capsule for lymphatic vessels Both men used a technique of random in jection of dye into the tissue This method results in hit or miss filling of the lym phatic vessels and frequently fills adjacent veins as well From figures in both reports it appears that capsular veins were also filled and the fluid backed up into the small cortical veins and the medullary sinuses In several of Sapin s figures he also appears to be labeling cortical sinu soids (sinusoidal capillaries fenestrated capillaries) as lymphatic vessels

The suprarenal glands examined microscopically in the present study were in jected with blue latex via the suprarenal vein This single discrete injection filled the central vein its tributaries medullary sinuses and the veins running out through the cortex and into the capsule The capsular lymphatic vessels were distinguished by the presence of valves and the presence of pink staining plasma instead of blue latex in their lumina (fig 4) The lym phatic vessels were followed through com plete serial sections of each gland in a search for tributaries from within the gland but none were found anywhere in the cortical or medullary parenchymal tissue

The arrangement of suprarenal gland lymphatics as reported in the present study is similar to that of the liver or kid ney Lymphatics of the former were de scribed by Mall (06) who found them in close company with the blood vessels in the interlobular tissue and never in association with the parenchymal tissue with in the liver lobule Of further note is Mall s observation that particulate matter in jected into the hepatic artery or portal vein quickly appears in the liver lym phatics

Clark and Clark (37) commented on Mall s classical study and stressed the point that a large proportion of lymph entering the thoracic duct (the liver being a major source of thoracic duct lymph) represents direct "leakage" from interlobu lar portal venules of fluid that has never

been in contact with cells of the liver lobule The Clarks using their rabbit ear chamber technique made a thorough study of the relation of lymph vessels to blood vessels They found veins and lymphatics lying side by side with no intervening tissue for long distances and verified Mall s observation that substances passed from vein to lymphatic In their experiments intravenously injected methylene blue quickly appeared in adjacent lymphatics and leucocytes and red blood cells passed from vein to lymphatic under conditions of chemical thermal and mechanical stimulus The Clarks noted too that in spite of this close association and easy passage of material from vein to lymphatic, open communication between the two types of vessels was never established

In contrast to these reports of transfer of material from vein to lymphatic but without open communication are reports of flow from lymphatic to vein and by way of very definite anastomoses Silvester ( 12) in a study of 25 new world species of monkey found constant communications between the intestinal and lumbar lymphatic channels on the one hand and the inferior vena cava or left renal vein on the other Interestingly enough he was unable to find any similar communications in 16 species of old world monkeys Job ( 18) found lymphaticovenous communications at the level of the iliac renal and subclavian veins in 48% of the rats he studied Job also noted an increase in the number of connections per animal under certain physiological conditions Blalock et al ( 37) demonstrated the development of lymphaticovenous anastomoses in dogs following ligation of the thoracic duct and found the greatest number of communications between the abdominal lymphatics and the inferior vena cava at the level of the renal veins Threefoot et al ( 63) obtained similar results after ligation of the cisterna chyli and found the development of anastomoses to be more rapid in animals pretreated with hexamethonium Threefoot et al ( 63) developed a method of radioisotope detection of lymphaticovenous connections and recorded their presence within five days of the occlusion of the thoracic duct in rats The intimate relation between lymphatic vessels and vasa vasorum of the inferior vena cava noted in the present study (fig 6) could well be the site for the development of anastomoses under the conditions mentioned above

From the foregoing reports it is evident that much remains to be learned about the lymphatics as a system and about their relation to the venous system In man it appears that lymphaticovenous connections are normally present only in the cervical region (thoracic duct and right lymphatic duct) However this arrangement can not be considered typical of all mammals since reports mentioned above clearly indicate the presence of lymphaticovenous communications in other areas of the body in a number of mammalian species Phylogenetic and ontologic studies of the lymphatic system similar to the early studies by Huntington and McClure ( 08) Huntington ( 08) and Sabin ('05) are badly needed to clarify this situation

The dynamics of the lymphatic system warrant additional investigation not only in the area of the development of new connections in response to various stimuli but also in regard to the dynamics involved in the transfer of material in the opposite direction from vein to lymphatic Finally the role of lymphatic vessels in structures such as the suprarenal gland where they are limited to the capsule and connective tissue elements requires additional study

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## PLATE 1

### EXPLANATION OF FIGURES

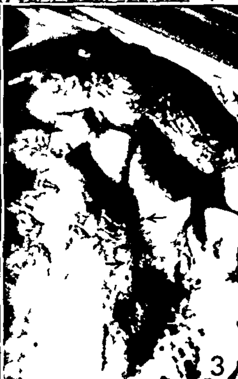
1 Subserous lymphatic vessels in the fascia overlying the suprarenal gland Mag 2 ×

2 Lymphatic vessels (arrows) from the suprarenal gland at right to a regional lymph node held by two hemostats

3 Several lymphatic vessels (arrows) passing from the right suprarenal gland to the dorsal surface of the inferior vena cava




2


3

## PLATE 1

### EXPLANATION OF FIGURES

1 Subserous lymphatic vessels in the fascia overlying the suprarenal gland. Mag. 2 ×

2 Lymphatic vessels (arrows) from the suprarenal gland [illegible] to a regional lymph node held by two hemostats.

3 Several lymphatic vessels (arrows) passing from the right suprarenal gland to the dorsal surface of the inferior vena cava.




2


3

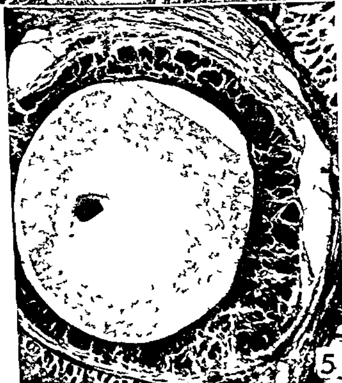
## PLATE 2

### EXPLANATION OF FIGURES

4 Suprarenal capsular vessels The thick walled arteries and veins filled with dark injection material are easily recognized The remaining vessels are lymphatics Note the close relationship between vein and lymphatic Mag 180 ×

5 The central vein containing injection material and surrounded by longitudinal muscle Two lymphatic vessels are seen external to the muscle A valve can be seen in the right lymphatic Mag 180 ×


4


5

## PLATE 3

### EXPLANATION OF FIGURE

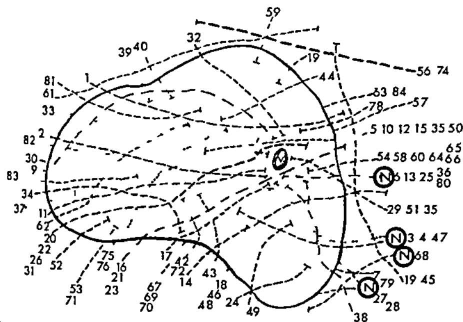
6 A section through the wall of the inferior vena cava A vas vasorum (V) is seen in two sections with a lymph vessel (L) sandwiched in between The vas vasorum opens into the vena cava at the upper right

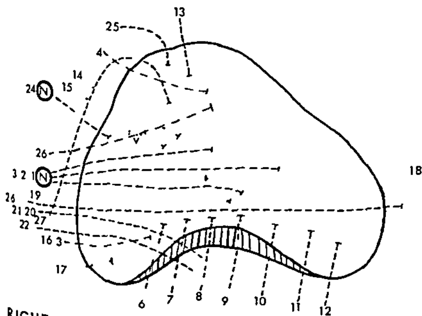

V
L
V
6

## PLATE 4

EXPLANATION OF FIGURE

7 All the lymphatic vessels seen in the study of 68 post mortem speci mens are charted and numbered consecutively on a single drawing of the ventral and dorsal surface of the right suprarenal gland This presentation indicates the areas where lymphatic vessels are most commonly found V suprarenal vein N lymph node


RIGHT VENTRAL


RIGHT DORSAL

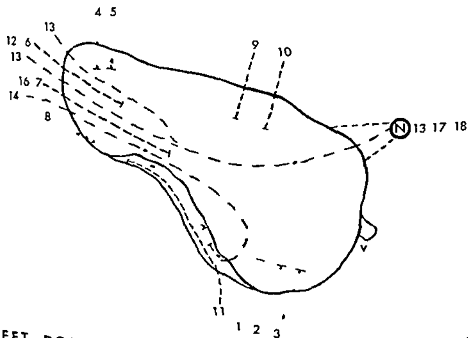
## PLATE 5

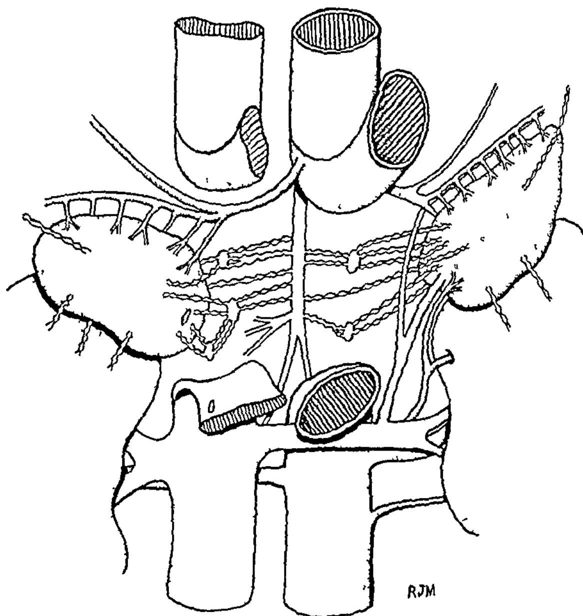
### EXPLANATION OF FIGURE

8 Same as for figure 7 but for the left suprarenal gland

71
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72 50 49 5
2
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4 N
63 59 57 40 22
64 41 23
56 65 60 52 42 24
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46 45 38 29 8
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LEFT VENTRAL






EXPLANATION OF FIGURE

9 An idealized drawing of the suprarenal gland lymphatic drainage based on observations of post mortem specimens

# The Synthesis and Storage of Protein by Isolated Lymphoid Cells, Examined by Autoradiography with the Electron Microscope [1]

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ABSTRACT Autoradiography with the electron microscope was employed to locate sites of synthesis and storage of newly formed protein in cells isolated from lymph nodes of immunized rabbits and incubated with tritium labelled leucine The chief object of the investigation was to locate the site of sequestration of newly formed antibody during the half hour that intervenes between its synthesis and release from the plasma cell By making counts of grains over nuclei Golgi complex and ergastoplasm and relating these to the relative volume of each region evidence was obtained that during the latent period of secretion antibody moves into the Golgi region from its site of synthesis in the ergastoplasm In analyzing the data some of the difficulties involved in quantitative autoradiography were considered and the statistical inappropriateness of using mean grain counts and their standard errors to estimate the concentration of isotope was discussed As an alternative with a sounder theoretical basis the ratio estimate was adopted and Fieller s theorem was used to obtain conservative estimates of confidence limits The results of statistical analysis supported the biological conclusion that newly formed antibody migrates to the Golgi region but the role of the Golgi complex in secretion of antibody remains to be explained

The hypothesis that plasma cells originate by differentiation from large lymphocytes received circumstantial support from the observation that among those cells active in synthesis of protein a series transitional in structure from large lymphocytes to mature plasma cells could be assembled

These experiments were undertaken to examine the mechanism of secretion of antibody by plasma cells While studying the secretion of antibody *in vitro* by cells isolated from lymph nodes of immunized rabbits Helmreich Kern and Eisen (61) observed a latent period of approximately 30 minutes intervening between the addition of radioactive amino acid to the culture medium and the first detectable release of labelled antibody from the cells This delay was not due to a slow rate of synthesis complete labelled antibody was recovered from cellular homogenates prepared during the latent period Further more binding of antibody to ribosomes — as newly formed albumin is bound in the liver (Peters 57) — did not account for the latent period most of the intracellular antibody recovered from homogenates was found free in the cell sap To test whether the delay was due to slow adaptation to the cultural environment C[14] labelled amino acid was added to cells already actively secreting tritium labelled antibody the usual latent period preceded release of C[14] labelled antibody even though secretion of tritium labelled antibody had continued unabated The process of secretion not only was orderly as demonstrated by these double labelling experiments it also proceeded to completion almost no intracellular labelled antibody remained 90 minutes after labelled cells had been washed and reincubated in a medium free of radioactive amino acid However when synthesis of protein was interrupted by the addition of puromycin the release of preformed antibody stopped short of completion (Helmreich Kern and Eisen 62) On the basis of these experiments the authors concluded that the secretion of antibody by plasma cells is a continuous orderly active process in which antibody moves from its site of synthesis to some other intracellular compartment for temporary storage prior to its release from the cell They also concluded that cellular dissolu

[1] Supported in part by grants GM 7176 and GM 3784 from the National Institutes of Health, United States Public Health Service
[2] Career Development Awardee United States Public Health Service

tion is not required to release antibody lysis would be expected to release intra cellular proteins indiscriminately but little labelled protein other than gamma globu lin accumulated in the incubation medium and the extracellular activity of the en zyme, aldolase did not increase during incubation

In the secretion of protein by pancreatic acinar cells and by chondroblasts similar latencies have been observed and the Golgi complex has been implicated as a site for temporary sequestration of the secretory product (Warshawsky Leblond and Droz, 63 Caro and Palade, 64 Jamieson and Palade 66 Revel and Hay, 63)

In the present experiments lymphoid cells were incubated with tritium labelled leucine and autoradiography was em ployed in a search for the site where labelled antibody is sequestered during the latent period of secretion Leucine labels all newly formed protein and thus could not be used to identify antibody di rectly but the intracellular distribution of immunoglobulin has been determined by immunohistochemical staining (Rifkind Osserman Hsu and Morgan 62 de Petris and Karlsbad 65) and lymphoid cells can be depleted of labelled antibody by re incubation in unlabelled medium (Helm reich Kern and Eisen 62) Therefore reincubation in unlabelled leucine was used to distinguish between immunoglobu lin and structural proteins in plasma cells The results can be interpreted to implicate the Golgi complex in sequestra tion of antibody but it has seemed neces sary to reevaluate the statistical basis for quantitative autoradiography at the level of the electron microscope in order to sup port such a conclusion

## MATERIAL AND METHODS

### *Immunization*

Male white rabbits weighing approxi mately 2 kg were injected in the hind footpads with an emulsion of antigen in Freund s complete adjuvant (Difco Lab oratories) In experiments I and II (table 1), the antigen was 5 mg of dinitrophenyl coupled bovine gamma globulin (DNP BGG) (Helmreich, Kern and Eisen 61)

In experiments III V, 60 mg of huma serum albumin (HSA) (Merck Sharp and Dohme) was injected with adjuvant both into the footpads and subcutaneousl over the scapulae

### *Cell suspensions*

Two to seven weeks after tion the rabbits were anesthetized sodium pentobarbitol (Nembutal Abbott) the popliteal lymph nodes excised and a suspension of cells wa prepared by teasing the nodes gently stainless steel rakes and stirring tl slowly for 1 5 to 2 hours at 0°C in oxygenated tris buffered balanced salt lution (Helmreich Kern and Eisen 61 After large fragments of tissue had removed by straining through gauze the suspended cells were wash three times by centrifuging at 100 × for ten minutes at 0°C and resuspend in fresh medium For incubation the were resuspended finally in a medium fered with phosphate and bicarbonate fortified with vitamins and amino acids but free of leucine In experiment V bit serum that had been heated to 56° for an hour was added to the medium a final concentration of 0 2% to more favorable conditions for cellu metabolism

At this point in the procedure of the cell suspension were tested for ability by staining with a dilute of eosin y a procedure that is said provide conservative estimates of viabil (Black and Berenbaum 64) The sions contained approximately 0 5 × cells/ml but accurate counts were not tempted because of clumping of the cell

### *Incubation (table 1)*

Three ml aliquots of the cell were incubated in siliconized 20 ml ers in a metabolic shaker at 37°C in atmosphere of 95% oxygen and 5% bon dioxide The cells were by gentle agitation for at least ten m before the addition of tritium labeled le cine Each beaker received 100 to microcuries of DL leucine 4 5 $H^3$ in specific activity from 3 5 to 5 5 curies millimol (New England Nuclear tion) The resulting concentration of le

TABLE I
*Experimental protocol*

| Experiment | Immunization | | | Incubation | | | | Autoradiography | |
|---|---|---|---|---|---|---|---|---|---|
| | Antigen | Immunizing interval | No. of rabbits | Group | Incubation period | Interval treatment | Reincubation period | Exposure | No of cells |
| | | *days* | | | *minutes* | | *minutes* | *days* | |
| | | | | 1 | 10 | | | 17 | 23 |
| | | | | 2 | 20 | | | 15 | 13 |
| | | | | 3 | 30 | | | 15 and 43 | 10 |
| | | | | 4 | 60 | | | 43 | 23 |
| | | | | 5 | 90 | | | 15 | 26 |
| I | DNP BGG | 50 | 6 | 6 | 120 | | | 43 | 20 |
| II | DNP BGG | 22 | 3 | 7 | 60 | P | 30 | | |
| | | | | 8 | 60 | P | 60 | 21 | 10 |
| | | | | 9 | 60 | C | 30 | | |
| | | | | 10 | 60 | C | 60 | 35 and 42 | 19 |
| | | | | 11 | 60 | PC | 30 | | |
| | | | | 12 | 60 | PC | 60 | | |
| | | | | 1 | 0.5 | | | 77 | 7 |
| | | | | 2 | 1 | | | 77 | 11 |
| III | HSA | 14 | 3 | 3 | 2 | | | 77 | 16 |
| | | | | 4 | 4 | | | 77 | 5 |
| | | | | 5 | 30 | | | 51 | 32 |
| | | | | 1 | 0.5 | | | 84 | 21 |
| | | | | 2 | 0.5 | C | 0.5 | 84 | 21 |
| IV | HSA | 14 | 2 | 3 | 0.5 | C | 1.5 | 84 | 14 |
| | | | | 4 | 0.5 | C | 3.5 | 84 | 26 |
| | | | | 5 | 0.5 | C | 29.5 | 84 | 16 |
| | | | | 3 | 10 | | | 9 | 14 |
| V | HSA | 23 | 3 | 4 | 10 | C | 180 | 19 | 15 |
| | | | | 1 | 90 | | | 17 | 20 |
| | | | | 2 | 90 | C | 180 | 9 | 22 |

Abbreviations DNP BGG dinitrophenyl-coupled bovine gamma globulin HSA human serum albumin P puromycin C unlabelled leucine

cine approximately 0 01 millimolar, was similar to that of the other amino acids present and presumably did not limit the rate of protein synthesis

Reincubation in unlabeled leucine was carried out by adding L leucine to the incubation medium in a final concentration of ten millimolar for experiments I, II and V and 0 03 millimolar in experiment IV In experiment V, the cells were washed once in leucine free medium before reincubation and NaCl was added to groups one and three in a final concentration of ten millimolar in order to simulate the osmotic effect of the leucine added to groups two and four

In experiments I and II puromycin was added in a final concentration of $3 \times 10^{-4}$ molar (Helmreich, Kern and Eisen 62) (Puromycin 2 HCl Lot 1326B 156 1 obtained through the generosity of Dr J M Ruegsegger of Lederle Laboratories and Dr J L Strominger of the Department of Pharmacology University of Wisconsin School of Medicine)

### *Fixation and imbedding*

Incubation was terminated by fixing the cells for 15 minutes at 0 C in 1% osmium tetroxide dissolved in incubation medium Fixed cells were dehydrated in ethanol and imbedded in epon 812 (Luft 61) using two different methods for incorporating the suspended cells into blocks suitable for dehydration and imbedding

1 In experiments I and II the incubation mixture was sedimented at 100 × g for five minutes at 0°C the medium decanted and the cells resuspended in the fixing solution After fixing ten minutes the cells were centrifuged again the fixative decanted and the cells resuspended in 1 ml of molten 3% agar agar The solidified agar was cut into small blocks for dehydration and imbedding

2 In experiments III V more rapid interruption of synthesis and better cytological preservation were achieved by diluting the incubating mixture to two volumes with cold 2% osmium tetroxide in incubation medium After fixing ten minutes the cells were sedimented into a hard pellet (10 000 × g for 5 minutes at 0°C) which could be dehydrated in toto and cut into small blocks for imbedding (Malame- 63)

### *Autoradiography*

For light microscopy sections 2 μ thi were cut with glass knives on a Port Blum microtome (Servall) mounted glass slides coated with chromated gel tin and dipped into diluted melted emu sion (Kodak NTB 3 Ilford L-4 or G-5 (Caro and van Tubergen 62) Dri slides were sealed in plastic boxes contai ing silica gel with a small piece of d ice added to retard fading of the la e image After exposure at room temper ture for 1 to 3 weeks autoradiograph were developed in Kodak D 72 and staine through the emulsion with ice-cold Giems stain (Benge 60) or with hot 1% azu B bromide

For electron microscopy thin sectio with pale gold interference color we mounted on collodion-coated copper grid and covered with a film of diluted Koda NTB 3 or Ilford L-4 emulsion accordin to the method of Revel and Hay (63) c of Caro and van Tubergen (62) Afte exposures of 2 to 12 weeks under cond tions similar to those described above th autoradiographs were developed in Koda Microdol X placed in 0 02 molar NaO for 30 minutes to digest away the gelati of the emulsion (Revel and Hay 63 and stained with lead hydroxide or urany acetate Electron micrographs were o tained with an RCA EMU 3 C electro microscope

### *Grain counts*

Crude estimates of radioactivity in a types of cells were made by countin grains in low power electron micrograph and recording the number/cell in nucleu nucleolus and cytoplasm Only cells co taining at least two grains were counte and because of irregularities in labellin unlabelled cells were not recorded

Grain counts in plasma cells were mad by photographing every plasma cell whe it was first encountered then choosin for counting only those that contained a least two grains showed no signs of de generation and possessed a recognizabl Golgi complex Electron micrographs wer made at a constant magnification of 5 40

X and enlarged to a final magnification of 21 600 X To avoid prejudice during the counting precedure sections of cells from several experimental groups were mixed and the identities of individual cells were not known Nevertheless obvious differences in total numbers of grains made some prejudice unavoidable Cells were subdivided into four regions

1 Nucleus (N) if present in the section

2 Golgi complex (G) demarcated from the surrounding cytoplasm by drawing a line on each micrograph to enclose smooth surfaced vesicles sacs and vacuoles but exclude all recognizable ribosomes and rough surfaced endoplasmic reticulum (fig 12)

3 The cytoplasm immediately surrounding the Golgi region (juxtagolgi or J) defined as an area extending the width of one grain or approximately 0 35 μ beyond the limits of G

4 The outer cytoplasm (O)

The cytoplasm exclusive of G (O and J taken together) was designated ER for its chief constituent ergastoplasm

The volumes of N G and ER were estimated by cutting electron micrographs into appropriate pieces weighing each region to measure its area and multiplying by an estimated section thickness of 0 08 μ Fourteen whole electron micrographs measuring 206 by 256 mm weighed from 9 364 to 9 583 gm with a mean of 9 421 gm and standard deviation of 0 0034 gm Therefore at a final magnification of 21 600 X each gm of paper represented approximately 1 $\mu^3$ of protoplasm

Statistical analysis was undertaken with the advice of Mrs Barbara Bartels Hixon of the Department of Preventive Medicine and Mr George Whitlow of the Computer Research Laboratory Washington University School of Medicine Computations were made at the Washington University Computer Facilities supported in part by grant G-22296 from the National Science Foundation

## RESULTS

### *Viability of cells*

Sixty to 80% of the cells failed to stain with eosin Y at the beginning of each experiment and a similar proportion remained apparently viable at the end of incubation Cells might have died and degenerated into unrecognizable debris during the course of incubation but Helmreich et al (61) obtained evidence that few cells degenerated in their experiments In any case only cells that showed no signs of degeneration were chosen for grain counts Therefore the results presumably represent the activities of viable cells

### *Morphology of cells*

Lymphocytes plasma cells monocytes macrophages granular leucocytes and erythrocytes could be recognized in electron micrographs by their resemblance to cells described previously by others (Bessis 61) (fig 7) Neither reticular cells nor connective tissue fibers appeared in the suspensions which were selected populations including only those cells that survived the process of isolation The term "large lymphocyte" will be used to designate all those large round basophilic cells with vesicular nuclei and prominent nucleoli but lacking ergastoplasm — variously called lymphoblasts plasmablasts hemocytoblasts or primitive reticular cells — because there seemed to be no adequate criteria for distinguishing between them in electron micrographs

Lymphocytes predominated in all preparations with small lymphocytes comprising 50 to 60% medium sized lymphocytes 20 to 25% and large lymphocytes 5 to 10% of the cells present Five to 15% were plasma cells and other types were scarce Two weeks after an injection of HSA (experiments III and IV) approximately 15% of the cells were large lymphocytes and 5% were plasma cells 3 to 7 weeks after an injection of DNP BGG (experiments I and II) these proportions were reversed

By arbitrary choice of electron micrographs a transitional series of cells from large lymphocyte to plasma cell could be assembled just as de Petris and Karlsbad (65) and Harris et al (65) have demonstrated Some of the large lymphocytes were in mitosis The most immature plasma cells could be distinguished from large lymphocytes only by the presence of ergastoplasm in the cytoplasm Mature plasma

cine, approximately 0 01 millimolar was similar to that of the other amino acids present and presumably did not limit the rate of protein synthesis

Reincubation in unlabeled leucine was carried out by adding L leucine to the incubation medium in a final concentration of ten millimolar for experiments I II and V and 0 03 millimolar in experiment IV In experiment V the cells were washed once in leucine free medium before reincubation and NaCl was added to groups one and three in a final concentration of ten millimolar in order to simulate the osmotic effect of the leucine added to groups two and four

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For electron microscopy, thin section with pale gold interference color wer mounted on collodion coated copper grd and covered with a film of diluted Kodak NTB 3 or Ilford L-4 emulsion accordin to the method of Revel and Hay (63) o of Caro and van Tubergen (62) After exposures of 2 to 12 weeks under conditions similar to those described above the autoradiographs were developed in Kodak Microdol X placed in 0 02 molar NaOH for 30 minutes to digest away the gelatin of the emulsion (Revel and Hay 63) and stained with lead hydroxide or uranyl acetate Electron micrographs were obtained with an RCA EMU 3 C electron microscope

*Grain counts*

Crude estimates of radioactivity in all types of cells were made by counting grains in low power electron micrographs and recording the number/cell in nucleus nucleolus and cytoplasm Only cells containing at least two grains were counted and because of irregularities in labelling unlabelled cells were not recorded

Grain counts in plasma cells were made by photographing every plasma cell when it was first encountered then choosing for counting only those that contained at least two grains showed no signs of degeneration and possessed a recognizable Golgi complex Electron micrographs were made at a constant magnification of 5 400

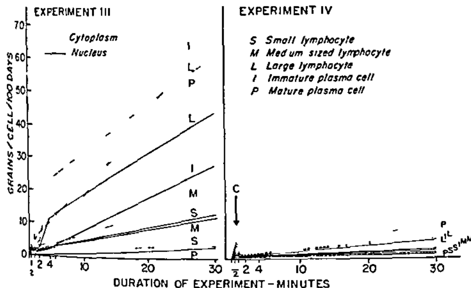



Fig 1 Average grain counts taken from low power electron micrographs of experiments III and IV expressed as grains per cell per 100 days autoradiographic exposure In experiment IV unlabelled leucine (C) was added to the incubation medium 30 seconds after the addition of tritium labelled leucine Apparently tritium labelled leucine was incorporated by all types of cells progressively for at least 30 minutes but the addition of excess unlabelled leucine interrupted this incorporation Large lymphocytes and plasma cells incorporated the largest quantities and — to judge by inspection of electron micrographs — the greatest concentrations of leucine but nuclei of mature plasma cells were relatively inactive

cytological preservation was inadequate for electron microscopy but the results of this experiment when assessed by light microscopy were comparable to those of experiment I Groups 7 9 11 and 12 of experiment I were omitted because the label seen by light microscopy in these groups accorded with predictions based on grain counts made in groups 8 and 10 and no new or critical information seemed forthcoming from the omitted groups

Some experimental groups were small the few plasma cells counted were all that could be found Scarcity of suitable cells can be attributed in part to the small proportion of plasma cells present in the cell suspensions to defects in preparation of autoradiographs and to the scarcity of silver grains in preparations incubated less than ten minutes even after prolonged autoradiographic exposure

Uncontrolled sources of variation in grain count — among the several experiments within individual experiments and even within individual autoradiographs — make direct comparisons of results risky Two different emulsions and several variants in the technique of their application were employed Among and within experiments the autoradiographic exposures required to produce usable grain counts varied widely creating possibilities for variation in fading of the latent image Within individual autoradiographs there were obvious variations in thickness of section and emulsion that were associated with variations in overall density of labelling Accordingly direct comparisons of grain counts between experimental groups were made with circumspection and reliance was placed as much as possible on comparisons of the several regions within each cell The statistical precautions taken to achieve these aims will be dealt with in the Discussion

The results (figs 2–6) can be interpreted to indicate that ergastoplasm began to incorporate leucine without delay but that within 10 to 30 minutes it reached a steady state in which the concentration of radioactivity varied little thereafter just as Mitchell has reported on the basis of

cells had condensed nuclei and no nucleoli Some plasma cells showed signs of degeneration, including condensed ergastoplasm, a reduced Golgi complex and a nucleus separated into two homogeneous phases differing in density (fig 8) Similar signs of degeneration can be found in plasma cells in intact lymph nodes (unpublished observations) and in thymic lymphocytes dying after an injection of hydrocortisone (Cowan and Sorenson 64) Therefore this probably represents the usual fate of effete plasma cells rather than an artifact of isolation

In most plasma cells ergastoplasmic sacs were relatively collapsed and only a few cells contained inclusions that could be interpreted as stored antibody These were of three types

1 Dilated ergastoplasmic sacs with contents of low density

2 Condensed ergastoplasmic contents (Russell bodies)

3 Dense bodies enclosed in smooth membranes lying near the Golgi complex (fig 12)

There were no obvious indications as to how antibody is released from plasma cells Signs of cytoplasmic shedding (the clasmatosis of Thiery 59) were not recognized but any such dynamic interpretation of static electron micrographs is difficult Some plasma cells did possess microvilli (figs 10–13) similar to those cited by Sorenson (64) to support his contention that antibody is released from peripheral ergastoplasmic sacs by what might be called reverse pinocytosis but we saw no other evidence to support his hypothesis There seemed to be no direct path of communication between the Golgi complex and the surface of the cell

Although a hypothetical transitional series from large lymphocyte to plasma cell could be assembled some macrophages also possessed features in common with immature plasma cells Most macrophages had condensed nuclei and cytoplasm containing few ribosomes but a few possessed vesicular nuclei with prominent nucleoli and concentrations of ergastoplasm in the peripheral cytoplasm (fig 9) Therefore the possibility that plasma cells differentiate from immature phagocytic cells should not be overlooked Indeed, the heterogeneous inclusions seen in some plasma cells may represent phagocytic debris (fig 12)

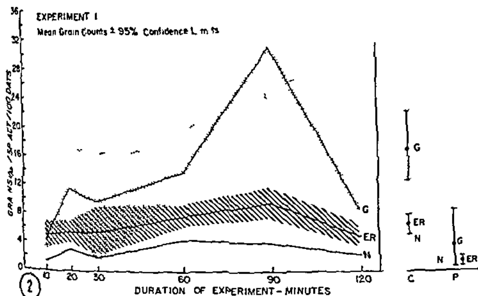
*Autoradiography*

As judged by both light and electron microscopy, all types of cells — including erythrocytes — incorporated radioactive leucine within 30 seconds after its addition to the incubation medium and continued to do so for at least 30 minutes (figs 1 7) Although differences in the degree of labelling among the various types of cells are exaggerated in figure 1 by differences in size of the cells it was apparent by both light and electron microscopy that large lymphocytes and immature plasma cells contained the greatest concentrations of radioactivity medium sized lymphocytes and mature plasma cells were intermediate and most small lymphocytes contained little or no label at autoradiographic exposures adequate for demonstrating radioactivity in other cells Macrophages with condensed nuclei contained little label, but those immature macrophages described above were labelled as heavily as medium sized lymphocytes (fig 9) Other types of cells incorporated leucine sparsely Among cells that labelled heavily nuclear radioactivity was prominent in all but mature plasma cells During the first ten minutes nuclear label was concentrated along the nuclear membrane and at the line of junction between light and dense chromatin by 30 minutes nucleoli were radioactive

Autoradiographic background as judged by the dearth of grains over regions of section containing only imbedding plastic was negligible A few grains lay over intercellular spaces (figs 7 9 10 11 13) but these may indicate either extracellular labelled protein or an occasional beta particle from intracellular tritium that penetrated more than half a micron before encountering a silver bromide crystal Cellular debris incorporated some leucine (fig 7)

*Quantitative autoradiography*

As indicated in table 1 material for grain counts in plasma cells was obtained from all but experiment II and selected groups of experiment I In experiment II




EXPERIMENT I

Ratio Estimate ± 95% and 99% Confidence Limits

G/ER

0 1 2 3 4 5 6

10 20 30 60 90 120 C P

3

DURATION OF EXPERIMENT - MINUTES

Figures 2 and 3

light microscopy (64b) The Golgi region incorporated little or no leucine during the first four minutes but had begun to accumulate radioactivity by ten minutes and within 20 minutes had surpassed the rest of the cytoplasm This sequence of events in ergastoplasm and Golgi region corresponds in timing to the course of synthesis intracellular accumulation and subsequent release of antibody described by Helmreich Kern and Eisen (61) In experiment V, the Golgi region was already labelled more heavily than the ergastoplasm after only ten minutes exposure to radioactive leucine but the addition of serum to the incubation medium made this experiment different from the others perhaps it accelerated the accumulation of newly formed protein in the Golgi region

Reincubation in unlabelled leucine produced varied results In experiment I after exposure to radioactive leucine for one hour it made little difference (figs 2 3) In experiment IV after only 30 seconds exposure to radioactive leucine the addition of unlabelled leucine — in a concentration only three times greater than that of the radioactive leucine — seemed to retard the incorporation of radioactive leucine by 30 minutes the label in the ergastoplasm was not as great as in experiment III and the Golgi region had not yet exceeded the ergastoplasm in radioactivity (figs 4 5) In experiment V in which serum was added to the incubation medium to improve conditions for the cells and in which the cells were washed to free them of radioactive leucine before the addition of a high concentration of unlabelled leucine both Golgi region and ergastoplasm were reduced by reincubation to the same low level of radioactivity (fig 6) As might be expected from the fact that antibody has not been identified in nuclei (Rifkind et al 62 de Petris and Karlsbad 65) nuclear label was not reduced by reincubation in unlabelled leucine

Puromycin reduced the label in both Golgi region and ergastoplasm, but counts in the Golgi region varied greatly from cell to cell and correlated poorly with counts in the ergastoplasm (figs 2 3)

## DISCUSSION

### *Cellular differentiation and the origin of plasma cells*

Our observations furnish indirect support for the contention that plasma cell originate from large lymphocytes (de Petris and Karlsbad 65) All the cells in the putative transitional series actively manu

Fig 2 Grain counts over plasma cells in experiment I The numbers of grains over nuclei (N) the Golgi region (G) and the remainder of the cytoplasm (ER) were related to the volume of each region estimated by weighing the appropriate areas cut from electron micrographs and the results were expressed as a ratio estimate (mean grains per region)/(mean volume of region) normalized to a specific activity of one curie of tritium per millimol of leucine and an autoradiographic exposure of 100 days Confidence limits were computed from Fieller s theorem (Cochran 63) Labelled leucine was added to all groups at the beginning of the experiment after one hour s incubation unlabelled leucine (C) or puromycin (P) was added to groups 10 and 8 respectively and the cells were reincubated for an additional hour It appears that grain counts in the Golgi region had exceeded those in the remainder of the cytoplasm as early as 20 minutes after the addition of labelled leucine and — except for the variable results at 30 minutes — remained higher thereafter Reincubation in unlabelled leucine seemed to have little effect but puromycin lowered grain counts generally However such comparative interpretations are subject to question because variations in thickness of sections and emulsion made obvious differences in autoradiographic efficiency — even within individual autoradiographs — and variations in exposure introduced possibilities for variation in fading of the latent image Therefore a statistical means was sought to make comparisons between regions within individual cells where variations in autoradiographic efficiency were minimal (See fig 3)

Fig 3 Intracellular comparisons of grain counts in the Golgi region (G) to those in the remainder of the cytoplasm (ER) in plasma cells of experiment I made cell by cell to avoid uncontrolled variations in autoradiographic efficiency and expressed as ratio estimates (see page 16) Fieller s theorem takes account of some skewness inherent in the distribution of some ratios therefore confidence limits are not always symmetrically related to the ratio estimates Arrowheads in place of upper 99% confidence limits indicate that the limits extend beyond the top of the graph The ratio of G to ER was significantly greater than 1 0 after incubation for 20 60 90 and 120 minutes and after reincubation in unlabelled leucine (C — group 10) but not after reincubation in puromycin (P — group 8) thus the results reinforce the tentative conclusions drawn from figure 2

EXPERIMENT III

Mean Grain Counts ± 95% Confidence Limits

EXPERIMENT IV

GRAINS/$\mu^3$/SP ACT/100DAYS

G ER C

DURATION OF EXPERIMENT MINUTES

4

EXPERIMENT III

Ratio Estimate ± 95% and 99% Confidence Limits

EXPERIMENT IV

$\frac{G}{ER}$

C

DURATION OF EXPERIMENT - MINUTES

5

Figures 4 and 5

factured protein, as judged by autoradiography, and not all of this new protein was removed by reincubation in unlabelled leucine presumably some of it was structural protein — the building material for cellular reproduction and differentiation Much protein was manufactured in the nuclei of immature cells of the series, but not in mature plasma cells — a parallel with Mitchell's observations on the synthesis of RNA ('64a '64b) Mitosis was observed in large lymphocytes All of these observations are consistent with the hypothesis that mature plasma cells are the effete end products of cellular differentiation from proliferating clones of large lymphocytes and immature plasma cells (Nossal '62) On the other hand, differentiation of plasma cells from phagocytic cells has not yet been ruled out If one takes a properly dynamic view of the question however then the categorization of cellular types on the basis of the morphology of dead cells — however detailed the analysis of that morphology may be — seems limited in its usefulness because of its failure to consider that any particular morphology may be only a passing phase in a rapidly and reversibly modulating population of cells

*Quantitative autoradiography*

Autoradiography with the light microscope was inconclusive in demonstrating the intracellular migration of antibody partly due to limitations in autoradiographic resolution but even more because it was difficult to distinguish Golgi region from ergastoplasm Nevertheless qualitative assessment by light microscopy supported the conclusions drawn from electron micrographs

Grain counts obtained from electron micrographs could be interpreted to indicate that antibody was synthesized in the ergastoplasm and migrated to the Golgi complex before being released from the plasma cell but before such conclusions can be defended from the biological viewpoint the chemical specificity of the method and the rationale for effective quantitation need to be established

1 *Chemical specificity* The object of the experiments was to identify newly formed protein There is strong evidence that cells incorporate leucine almost exclusively into new protein (Caro and Palade '64), and that after the usual preparation of tissue for autoradiography more than 90% of the remaining radioactivity represents protein (Warshawsky Leblond and Droz '63), but Caro and Palade ('64) discovered that when tissues were fixed in osmium tetroxide as much as 4% of the free amino acid present at the time was bound to the tissue in such a way that it survived preparation for autoradiography and produced silver grains

Thus except in groups reincubated in unlabelled leucine, some of the label seen in the present experiments might have represented free leucine rather than newly formed protein — this could account for the few silver grains seen over erythrocytes, as noted by Caro and Palade ('64) If the intracellular distribution of amino acids was relatively uniform as suggested by the autoradiographic experiments of Benditt et al ('65) then the spurious background thus produced might have obscured regional differences in the concentration of labelled protein but it would not have created false differences On the other hand if recently acquired amino acid failed to mix freely with the

Fig 4 Grain counts over the Golgi region (G) and the remainder of the cytoplasm of plasma cells in experiments III and IV expressed as in figure 2 Groups 1 to 4 were combined to obtain values for incubations of one-half to four minutes inclusive In experiment IV, unlabelled leucine (C) was added 30 seconds after the addition of tritium labelled leucine During the first four minutes incubation label in the ergastoplasm appeared to exceed that in the Golgi region whereas the reverse was true by 30 minutes after addition of labelled leucine unlabelled leucine seemed to reduce the rate of incorporation of label and erase the differences between regions However the objections raised in relation to figure 2 apply to these interpretations as well Intracellular comparisons are needed

Fig 5 Intracellular comparisons between grain counts in the Golgi region (G) and those in the remainder of the cytoplasm (ER) expressed as in figure 3 Groups for one half to four minutes incubation were combined and unlabelled leucine (C) was added to experiment IV as in figure 4 Using Fieller's theorem confidence limits are derived from the roots of a quadratic equation if imaginary roots are obtained as occurred for the upper 99% limits after one half and four minutes incubation in experiment III no limit can be estimated The results of these intracellular comparisons reinforce the conclusions drawn from figure 4

gens Therefore it seems unlikely that bound leucine was a source of erroneous localization of protein accordingly the reduction of label in Golgi region and ergastoplasm produced by reincubation in unlabelled leucine in experiment V will be interpreted as excretion of labelled immunoglobulin

2 *Resolution* Caro (62) estimated that the resolution for tritium in electron micrographs fell between 0 1 and 0 5 μ depending upon experimental conditions The Golgi complex in our micrographs had a minimum width of 0 7 μ therefore label ling of the Golgi complex should be distinguishable from radioactivity in the surroundin cytoplasm but the spread of beta particles across the boundaries of such a small region could distort grain counts However most Golgi complexes were wider than 0 7 μ and in any case errors of resolution would tend to obscure true differences in radioactivity between adjacent regions rather than creating false differences For example the few grains seen over the Golgi region after incubation for less than ten minutes might have arisen from tritium in the "juxtagolgi cytoplasm if so then the true difference between Golgi complex and ergastoplasm was greater than it seemed

Another problem of resolution is not so easy to rationalize Many grains occurred along the nuclear membrane and were included in the counts for nuclei or ergastoplasm depending upon their exact location de Petris and Karlsbad (65) found concentrations of antibody within the cavity of the nuclear membrane a space continuous with the cavities of the ergastoplasm. If silver grains along the nuclear membrane represented such antibody then inclusion of some grains in the counts for nuclei would have produced a falsely low estimate of the radioactivity in ergastoplasm and exaggerated the extent to which the Golgi complex appeared to exceed the ergastoplasm in label after prolonged incubation However recounting a group of autoradiographs to include all such grains in the ergastoplasm increased the average count by less than 4% such an error is too small to compromise the observed differences (fig 2)

3 *Quantitation* The basis for quantitation of autoradiographs lies in the presumption that grain counts are proportional to the total quantity of radioisotope present in the specimen This will be true only if the specimen is thin enough for a representative proportion of beta particles to reach the emulsion and if all other factors affecting the probability that grains will form are held constant The adaptation of autoradiography to the electron microscope has been haunted by the fear that constancy of conditions would need to be sacrificed for improvement in resolution This ghost has been laid by recent work Techniques have been standardized (Caro and van Tubergen 62 Salpeter and Bachmann 64) to such an extent that reproducible quantitative results can be obtained Caro and Shnos (65) obtained reliable estimates of efficiency for tritium but they pointed out its great sensitivity to variations in thickness of the emulsion Bachmann and Salpeter (65) looked for non linearity of response by testing for fading of the latent image in the emulsion during prolonged autoradiographic exposure They reported that fading did not occur in Ilford L-4 emulsion exposed at room temperature in dry air for 2 months but others have not been so fortunate and the factors that control fading are poorly understood Thus reproducible autoradiographic efficiency is achievable but definitive methods for maintaining conditions constant and controlling fading of the latent image have not yet been developed In the present experiments obvious variations in thickness of sections and emulsion made visible differences in efficiency and some exposures exceeded two months Therefore constant efficiency cannot be taken for granted when comparing different experimental groups different autoradiographs within one experimental group or even different plasma cells within individual autoradiographs A statistical method is needed for dealing with the large variability in the data and for making intracellular comparisons

Before statistical analysis can be attempted there must be some assurance that the data were collected from samples that because they were randomly chosen truly represented the phenomena under

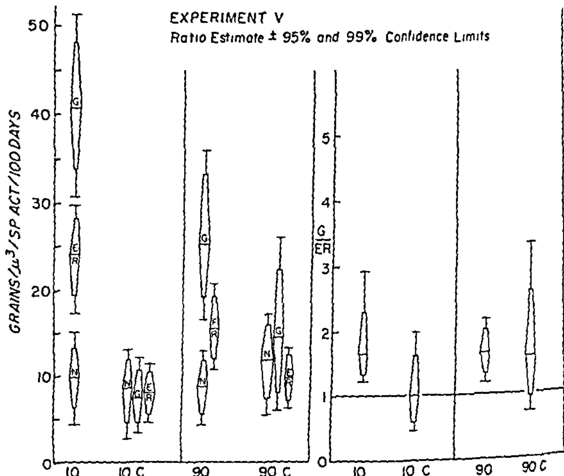



Fig 6 Grain counts over plasma cells in experiment V expressed both as grain counts for each region as in figure 2 and as the ratio of grain counts in the Golgi region to those in the ergastoplasm (ER) made cell by cell as in figure 3 After incubation in labelled leucine for 10 or 90 minutes the cells were washed resuspended in unlabelled leucine and reincubated for three hours (10 C and 90 C) Grain counts in the Golgi region had already exceeded those in the ergastoplasm after only ten minutes incubation but this experiment differed from the others in that rabbit serum was added to the incubation medium to improve conditions for the cells perhaps this accelerated the accumulation of newly formed protein in the Golgi region Reincubation in unlabelled leucine reduced the label in both Golgi region and ergastoplasm but made little difference in nuclei (N) These observations are consistent with the reported identification of immunoglobulins in ergastoplasm and Golgi complex by immunohistochemical methods and they are interpreted to indicate that the label removed by reincubation in unlabelled leucine was newly formed immunoglobulin If this interpretation is correct then antibody not only accumulates in the Golgi complex it also is removed from there in the course of secretion

total intracellular pool as proposed by Kipnis Reiss and Helmreich (61) then local concentrations of labelled leucine might have been a source of confusion However, there is indirect evidence in the experimental results that such confusion was not a source of serious error First intracellular label was low during the first four minutes of incubation, when labelled leucine should already have entered the cells in high concentration (Kipnis Reiss and Helmreich 61) Second reincubation in unlabelled leucine by making labelled leucine relatively unavailable should have reduced any spurious label due to bound amino acid but nuclear label was never reduced by this procedure and in experiment I label was not reduced in the Golgi region or ergastoplasm either In contrast when cells were reincubated in puromycin which presumably interrupted the synthesis of protein but did not change the availability of labelled leucine label appeared to be reduced in all three re-

distribution (Snedecor 56) Hanawalt et al (61) demonstrated that this was true for whole cells of uniform size and specific activity and Caro and Shnos (65) used Poisson means to express grain counts over sections of uniformly labelled bacteria in electron micrographs In large samples Poisson distributions approach normality Kastenbaum and Hanna (65) studying mixed populations of whole cells assumed that their data were distributed normally and used analysis of variance to test the significance of differences between mean grain counts However Stewart et al (65) demonstrated that small errors in estimation of background skew the distribution of grain counts over whole cells and they adopted the "grain median as a more reliable estimate of radioactivity than the mean grain count

In electron micrographs background was negligible but the use of sections introduces other difficulties both for light and electron microscopy Poisson statistics are based on specimens of uniform size but intracellular regions in sections are variable in size This difficulty could be circumvented by subdividing each region into uniform areas by superimposition of a standard grid on the electron micrograph but the *Golgi* region was too small and irregular in shape to be subdivided with any accuracy

Another approach can be based on the statement that in homogeneously labelled regions where the probability that grains will occur is proportional to the total radioactivity present grain counts should be proportional to the volume of the region In other words if grain count is plotted against volume of the region for a homogeneously labelled sample of regions the graph should describe a straight line that passes through the origin and the slope of the line should be a measure of the concentration of radioactivity in the specimen Schultz et al (65) found that such linear regressions adequately described the distribution of grain counts over nuclei in sections from mice injected with tritium labelled amino acid and they concluded that this indicated a uniform specific rate of protein synthesis per unit volume of nucleus Our data also fit such linear regressions but with considerable variation

Thus the ratio of grain count to volume seems a reasonable estimate of the concentration of radioactivity but the average of such ratios which other authors have used for statistical comparisons is not the best estimate when groups are small and grain counts are low This is because in an average of the ratios all specimens in the sample — regardless of volume — receive the same weight Where counts are low and many counts of zero occur as in the Golgi region after short incubation periods this error in weighting can be particularly serious a count of zero in a large volume is a more impressive indication of low radioactivity than a count of zero in a small volume but both would influence an unweighted average equally What is needed is a weighted average the ratio estimate is such a weighted average that provides theoretically unbiased values for the slopes of linear regressions which pass through the origin (Cochran 63) The ratio estimate is calculated as the ratio of total grain count for a sample to total volume of the sample or — what is the same thing — the ratio of mean grain count to mean volume

The difficulty with using ratio estimates is that there is no exact method for calculating variance because ratios by nature are not normally distributed In large samples the approach to normality may be close enough to allow use of an approximate "normal" solution but in small samples where the coefficient of variation of the denominator (volume) exceeds 0 1 as in most of our experimental groups estimated variances would be misleadingly small In this event Fieller s theorem still provides an approximate estimate of confidence limits for the ratio estimate it takes some account of the natural skewness in distribution of ratios and it is relatively safe for use with small samples

Therefore ratio estimates and confidence limits for grain counts were computed and selected results are presented graphically in figures 2 4 and 6 Although there seemed to be significant differences between Golgi region and ergastoplasm in many experimental groups differences were not clear after brief incubation peri

study and were not biased by some artifact of selection In order to achieve randomness of selection all recognizable plasma cells were photographed as soon as encountered and from these all were chosen for counting that contained at least two grains (but not so many that accurate counting was impossible) had a visible Golgi complex and showed no signs of degeneration Thus subjective judgements were required for selecting cells for counting other real or potential sources of nonrandom selection also operated Single cells may have appeared in successive sections and been counted twice The choice of cells with visible Golgi complex although necessary for intracellular comparisons, led to greater representation of the juxtagolgi cytoplasm than of the outer cytoplasm Accidental coincidence of grains may have reduced the apparent grain count in heavily labelled cells (fig 11) but to have eliminated all possibility of coincidence would have been to reduce the size of the samples too much Errors of resolution in which grains over one region arose from tritium in an adjacent region would have operated more in heavily labelled than lightly labelled cells however as pointed out above these errors would tend only to obscure true differences in concentration rather than creating false differences In addition to these non random sources of selection heterogeneity of the material may have been a source of bias Plasma cells varied in maturity, and the relative numbers of mature and immature plasma cells varied from experiment to experiment The Golgi complex was not homogeneous in structure if the distribution of label was similarly heterogeneous then thin sections of Golgi complex may not all have represented the same population of labelled protoplasm Thus there were several real or potential sources of bias in the sampling procedure None seems — from the theoretical point of view — large enough to compromise statistical analysis but it will be important to seek independent reassurance that the results are logically consistent As evidence for this results obtained under similar conditions in different experiments should be comparable and results obtained under varying conditions within individual experimen should vary in an appropriate fashion

The method used for expressing gra counts imposes limits on the conclusio that can be drawn When expressed as t proportional distribution of grains several compartments, the data may vide evidence as to the timing of chang in distribution but it allows no concl sions concerning the concentrations tope present (Caro and Palade, 64) measuring the area of each compartme in the section relative concentrations isotope can be estimated and changes small compartments can be detected wit greater sensitivity, as pointed out by Ro and Benditt (65)

Thus there is the basis in previous wor for estimating the concentration of isotop by counting the grains per unit volume o protoplasm according to the following ex pression Concentration of isotope (grains/area of region/thickness of sec tion/exposure) × efficiency × error Erro would include variations in efficiency du to inconsistencies in thickness of sectio and emulsion non linearity due to fadin of the latent image and other unknow factors Because autoradiographic effi ciency was not measured in the presen experiments, only relative concentration of isotope can be estimated

4 *Statistical analysis* There is as ye no firmly established rationale for analysi of grain counts over sections of heter geneous cells or tissues Many investig tors have avoided statistical testing alt gether others have calculated mean grai counts and standard errors and have use the t test to evaluate the significance differences without discussing the ratio ale for their use (Warshawsky Leblon and Droz 63 Ross and Benditt 65 The use of these statistics presuppos normal distribution of the data b neither *a priori* reasons for expecting normal distribution nor tests to demo strate normality have been presented O data were not normally distributed judged by graphic methods

Theoretically with specimens of un form size and radioactivity the small b uniform probability that grains will occ over any individual specimen should r sult in grain counts that follow a Poisso

formed protein had accumulated in the Golgi region In attempting physiological interpretation of these conclusions three questions will be discussed 1 What part of the newly formed protein is antibody or immunoglobulin? 2 What is the source of the protein that accumulates in the Golgi region? 3 What role does the Golgi complex play in the secretion of antibody?

1 *Identification of antibody* Judging from the results of reincubation in unlabelled leucine both ergastoplasm and Golgi region contained newly formed antibody but there was little or none in nuclei. Immunohistochemical staining has led to similar conclusions (Rifkind et al 62 de Petris and Karlsbad 65) On the other hand some of the label in ergastoplasm and Golgi region was not removed by reincubation in unlabelled leucine This label may represent structural protein or it may be due to antibody that was not released because of cellular injury as discussed above

2 *The source of label in the Golgi region* Ribosomes were not seen within the Golgi complex and the label there was low during the first 4 to 10 minutes of incubation Others have concluded that the Golgi complex in the pancreas does not synthesize protein (Warshawsky et al 63 Caro and Palade 64) and a similar conclusion seems warranted for plasma cells Therefore the label that accumulated in the Golgi region and was removed by reincubation in unlabelled leucine presumably represents antibody that had migrated to the Golgi region after synthesis in the ergastoplasm Jamieson and Palade recently have obtained evidence for such a migration in the pancreas (66) If the label not removed by reincubation was structural protein its source might have been either ergastoplasm or nucleus

3 *Role of the Golgi complex in secretion* The foregoing evidence can be interpreted to indicate that antibody migrates from the ergastoplasm to the Golgi region during the latent period of secretion and that it reaches a high concentration there just at the time when the concentration of labelled antibody in the ergastoplasm reaches a plateau and antibody first appears extracellularly (Helmreich Kern and Eisen 61) Therefore in the plasma cell as elsewhere the Golgi region is by implication a way station on the route of secretion

However the "role" of the Golgi complex in secretion of antibody is not yet obvious In the pancreas the Golgi complex seems to be the site where secretory material is concentrated into zymogen granules but few plasma cells contain anything that might be called secretory granules — Russell bodies lie within the ergastoplasm not in the Golgi complex Synthesis of carbohydrate has been attributed to the Golgi complex (Peterson and Leblond 64) perhaps the carbohydrate moiety of antibody (Cohen and Porter 64) is manufactured there Whether continued synthesis of protein is necessary for the Golgi complex to play its role in secretion was not determined by our experiments because puromycin produced equivocal results If the label remaining in the Golgi region after reincubation in unlabelled leucine was structural protein it may be an indication that the Golgi complex serves as a depot for membranes involved either in the formation of ergastoplasm or the release of antibody from the cell

The mechanisms for release of antibody remain as obscure as the role of the Golgi complex As mentioned in RESULTS the Golgi complex is remote from the surface of the cell and no morphological indications of transport of antibody from the Golgi region or its excretion from the cell were detected Even when all this is understood it will still be necessary to explain what mechanisms coordinate synthesis storage and excretion — why some plasma cells accumulate antibody in dilated ergastoplasmic sacs or in condensed Russell bodies whereas most plasma cells seem to excrete antibody as fast as it is manufactured

In other words more work needs to be done to understand the secretion of antibody This study has produced evidence of the importance of the Golgi complex in the process and has demonstrated some of the possibilities and difficulties of quantitative autoradiography with the electron microscope Future work might profitably be concerned with synthesis of membranes and of carbohydrates by plasma cells

ods and as pointed out earlier, *direct* comparisons had to be made with caution because of variations in the thickness of sections and emulsion

Therefore more reliable estimates were sought in intracellular comparisons where thickness of section and emulsion were relatively constant To achieve such comparisons as for example between Golgi region and ergastoplasm ratio estimates were constructed as follows

$$\frac{{}^{G}\text{grains}/{}^{G}\text{volume}}{{}^{ER}\text{grains}/{}^{ER}\text{volume}}$$

However in order to give proper weight to Golgi regions in which no grains were found and to make comparisons cell by cell the ratio estimates actually were computed by isolating Golgi grains in the numerator as follows

$$\frac{{}^{G}\text{grains}}{{}^{G}\text{volume}/{}^{ER}\text{grains}/{}^{ER}\text{volume}}$$

This seemed justifiable because volume could be considered as a given quantity rather than an independent variable and therefore both numerator and denominator each contained only one independent variable These intracellular comparisons some of which are presented in figures 3 5 and 6 supported the conclusions drawn from individual ratio estimates and, in addition indicated that the Golgi region was significantly lower in label than the ergastoplasm after incubation for one half to four minutes

These statistically significant results when examined for logical consistency were for the most part reproducible among the several experiments and varied with differing experimental conditions in ways that fit biological expectations Therefore it will be presumed either that selection of samples was adequately random or that selective biases operated so uniformly in all experiments that the results are true only for some particular segment of the population of plasma cells In either case biological interpretation seems justified

*Biological interpretation*

Before interpreting the results in terms of normal cellular physiology the possibility needs to be considered that the mechanisms of secretion were altered either by the damaging effects of radiati[on] from tritium or by unfavorable conditi[ons] of incubation This possibility applies p[ar]ticularly to those experimental grou[ps] which underwent prolonged incubation [or] reincubation It might explain for [in]stance the fact that in experiment I gra[in] counts were lower after two hours inc[u]bation (group 6) than after incubation for one hour (group 5) (fig 2) That t[ri]tium may damage cells is adequately a[t]tested by Wimber s review ( 64) but m[ost] of the experiments he cites involved th[y]midine and damage was indicated [by] reduction in mitotic rate there is litt[le] evidence from which to evaluate th[e] damaging effects on secretory process[es] of short exposures to tritium labelled am[i]no acids Helmreich et al ( 61) reporte[d] that synthesis and release of antibod[y] continued unabated for at least five hours but the concentrations of tritium used i[n] the present experiments were as much a[s] 2 5 times greater than they used and mu[st] also have been considerably greater tha[n] those to which cells have been exposed i[n] studies of secretion *in vivo* (Caro an[d] Palade 64) However, the cells used fo[r] grain counts in our experiments showe[d] no visible signs of damage at the end o[f] incubation — as seen in electron micro graphs — and they failed to stain wit[h] eosin Y The reduction of grain counts i[n] both Golgi region and ergastoplasm upo[n] reincubation in unlabelled leucine in ex periment V where the greatest care wa[s] taken to insure favorable conditions fo[r] incubation can be taken as evidence tha[t] mechanisms for release of antibody con tinued to operate perhaps the failure of grain counts to diminish with reincuba tion in experiment I group 10 is evidence that in this group mechanisms of release had been damaged Therefore although morphological evidence and dye exclusion indicated that the cells were undamaged altered mechanisms of secretion must re main as possible explanations for the results

On the basis of all the previous consid erations it seems appropriate to conclude that incorporation of leucine into new pro tein began without delay in nuclei and ergastoplasm — but not in the Golgi re gion — and that within 20 minutes newly

protein had accumulated in the Golgi region In attempting physiological interpretation of these conclusions three questions will be discussed 1 What part of the newly formed protein is antibody or immunoglobulin? 2 What is the source of the protein that accumulates in the Golgi region? 3 What role does the Golgi complex play in the secretion of antibody?

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ACKNOWLEDGEMENT

I wish to express grateful appreciation to Dr Ernst Helmreich, of the Department of Biological Chemistry Washington University School of Medicine, who called my attention to the usefulness of this experimental system for studying secretion of antibody, carried out immunization, isolation of cells and incubations in experiments I and II and served as a valuable source of advice and help throughout the course of the work

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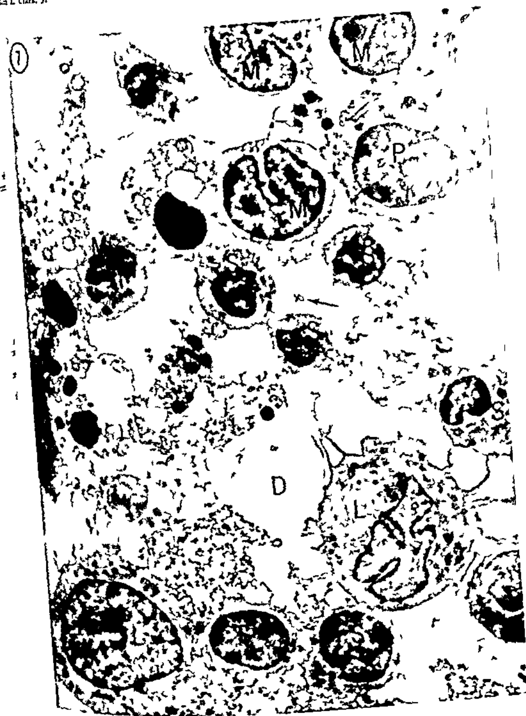
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Figures 7–13 are electron micrographs and autoradiographs of cells isolated from popliteal lymph nodes of immunized rabbits and incubated with tritium labelled leucine Sections were stained with uranyl acetate after removing gelatin of the photographic emulsion with NaOH

## PLATE 1

### EXPLANATION OF FIGURE

7 These cells were incubated 90 minutes in labelled leucine (experiment V group 1) and the autoradiograph was exposed for 18 days every cell is labelled Lymphocytes — identified as small (S) medium sized (M) and large (L) — predominate one plasma cell (P) is present and the remainder is debris — presumably derived from cells that degenerated during the course of isolation or incubation The plasma cell with its large vesicular nucleus and prominent nucleolus was designated as immature there is label in both nucleus and cytoplasm The large lymphocyte in the lower left is typical of those large basophilic cells with vesicular nuclei and prominent nucleoli that stand at the beginning of the hypothetical transitional series leading from lymphocytes to plasma cells The large lymphocyte in the lower right with contorted nucleus and narrow pseudopods has the appearance of becoming a macrophage The medium sized lymphocyte on the left appears to be in mitosis The large lipoid droplet (D) contains two silver grains similar labelling over erythrocytes where synthesis of protein is equally unexpected was taken as evidence that a small quantity of free leucine was bound to cells and debris by fixation in osmium tetroxide as reported by Caro and Palade ( 64) The few silver grains seen over intercellular spaces (arrow) probably do not represent background in view of the observation that areas of imbedding plastic outside the pellet of cells were free of grains perhaps the intercellular label represents newly formed antibody of other protein released from the cells × 1500

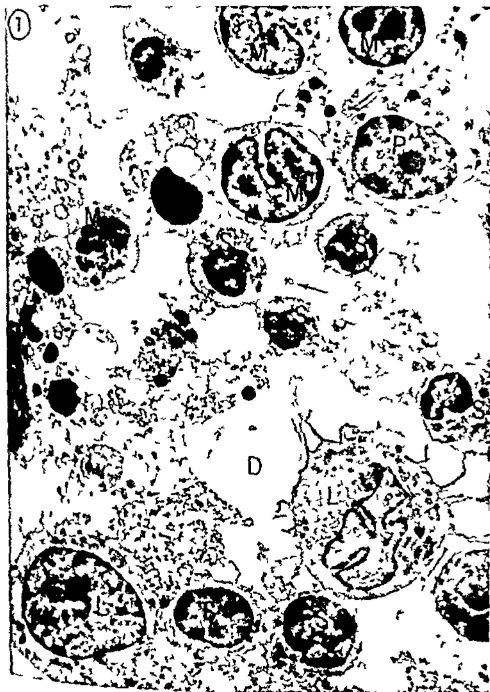

1
M
M
P
M
S
S
D
L
S

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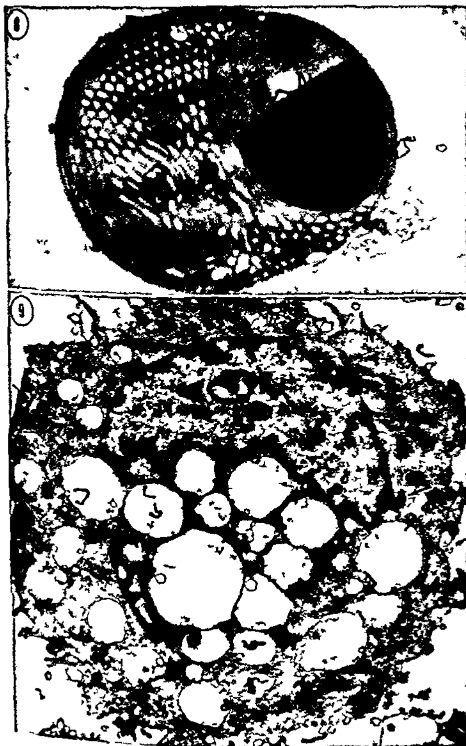

1
M
M
P
M
M
S
S
S
D
L

## PLATE 2

### EXPLANATION OF FIGURES

8 This plasma cell incubated ten minutes in labelled leucine (experiment V group 3) and exposed for nine days is typical of those described in the text as degenerating or senescent The nucleus (N) has separated into two phases and the cytoplasm consists chiefly of condensed ergastoplasm Although this cell seems relatively active in the synthesis of protein many such cells were not The mottled appearance of the intercellular space probably indicates the original spacing of silver bromide crystals in the emulsion × 15 000

9 This large cell appears to have been actively ingesting membranous debris along with large quantities of incubation medium It is typical of one type of macrophage seen in these preparations characterized by a large vesicular nucleus (N) prominent nucleoli cytoplasmic accumulations of ergastoplasm (arrows) and moderately heavy label in both nucleus and cytoplasm Perhaps this type of cell is a progenitor of plasma cells (Experiment V group 3 incubated ten minutes exposed 47 days) × 12 000

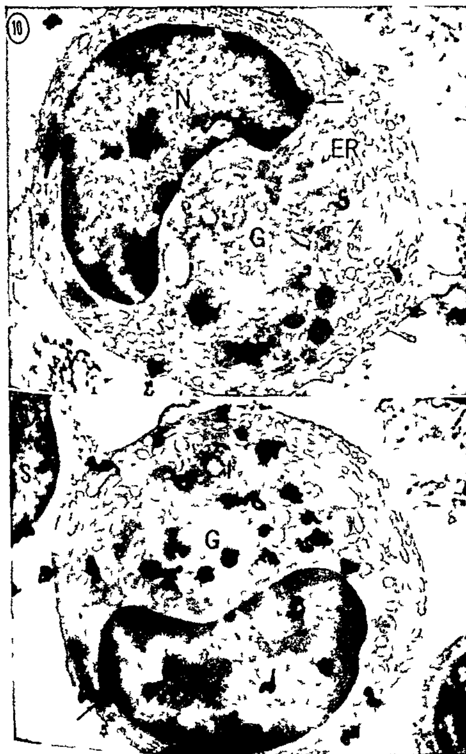

8
9

## PLATE 3

### EXPLANATION OF FIGURES

10 This plasma cell was incubated in labelled leucine for ten minutes and reincubated in unlabelled leucine for three hours (experiment V group 4, exposed 20 days) There are three grains over the nucleus (N) two over the Golgi region (G) and seven over the remainder of the cytoplasm (ER) The grain in the upper left lies more than one grain s width (approximately 0 3 $\mu$) beyond the edge of the cell and was considered extracellular The dense material at the arrow was identified as dirt rather than a silver grain In terms of the concen tration of grains N = 2 4 G = 3 6 and ER = 3 1 grains/$\mu^3$/sp act / 100 days × 15 000

11 This plasma cell was incubated 90 minutes in radioactive leucine (experiment V group 1 exposed 17 days) The nucleus contains eight grains the Golgi region (G) six grains and the remainder of the cytoplasm 21 grains The large grain at the arrow may be two grains superimposed and thus represent a source of error in counting heavily labelled cells In terms of concentration N = 7 3 G = 28 0 and ER = 10 2 grains/$\mu^3$/sp act /100 days The cytoplasm contains a dense heterogeneous inclusion (I) (S small lymphocyte) × 15 000


10
N
ER
S
G
S
G

## PLATE 3

### EXPLANATION OF FIGURES

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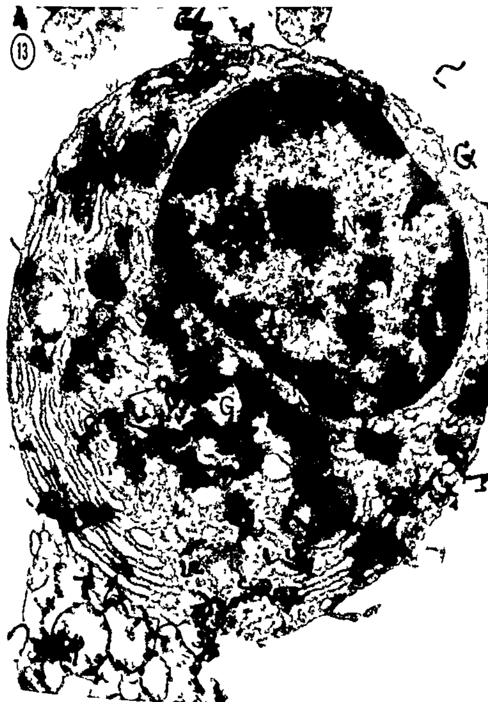
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19
G

## PLATE 4

### EXPLANATION OF FIGURE

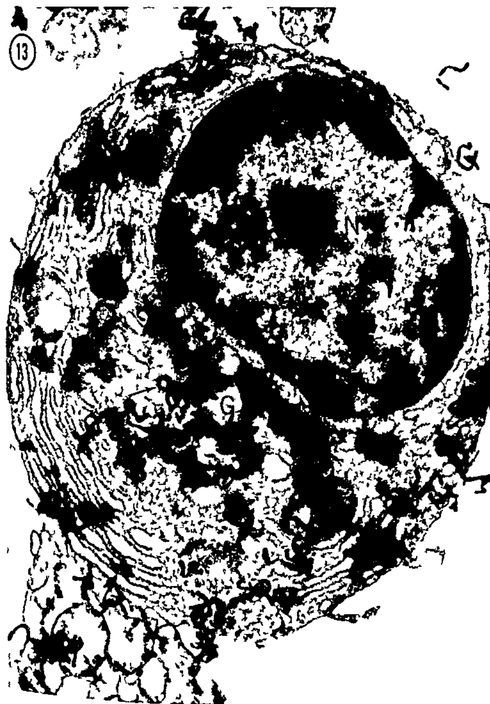
12 An enlargement of figure 11 marked to indicate how the Golgi region (G) was delimited from the ergastoplasm for grain counts As can be seen subjective judgement was important in drawing this line In this electron micrograph five grains lie entirely within the Golgi region and two others lie on the boundary one was assigned to the Golgi region and the other to the ergastoplasm Five grains were considered to lie within one grain s width of the Golgi region and were designated as juxtagolgi The dense cytoplasmic inclusion (I) is enclosed by a smooth surfaced membrane and resembles inclusions in macrophages × 27 000


13
G
N
G

## PLATE 5

### EXPLANATION OF FIGURE

13 This plasma cell was incubated ten minutes in labelled leucine in an incubation medium containing rabbit serum (experiment V group 3 exposed 47 days) In this over exposed autoradiograph the concentration of newly formed protein in the Golgi region (G) and the relatively sparse labelling of the nucleus (N) can be appreciated by inspection (Arrow centriole) × 21 000


13
N
G
G

# Dual Innervation of the Mammalian Urinary Bladder [1]

## A HISTOCHEMICAL STUDY OF THE DISTRIBUTION OF CHOLINERGIC AND ADRENERGIC NERVES

AHMAD EL-BADAWI [2] AND ERIC A SCHENK

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ABSTRACT The pattern of autonomic innervation of the urinary bladder was studied in the cat dog rabbit and rat using specific histochemical technics for acetylcholinesterase and norepinephrine Cholinergic and adrenergic ganglion cells exist in all layers throughout the bladder wall Large cholinergic and adrenergic nerve trunks coursing in the adventitial coat and deep lamina propria branch into the muscularis The terminal cholinergic ramifications form a neuroterminal plexus which surrounds every smooth muscle cell in the bladder wall The terminal adrenergic fibers are less abundant do not form a plexus and show regional variations in number at different levels and depths of the muscularis These variations suggest that two regions of the bladder namely the base and body may be distinguished on the basis of differences in muscular innervation

In the lamina propria cholinergic and adrenergic nerves are grouped as deep and superficial subepithelial nerves The latter form networks of variable complexity and supply fibers to the epithelium Throughout the bladder wall the blood vessels have a dual cholinergic and adrenergic perivascular plexus from which fibers extend into the media

Although the basic pattern of innervation is similar in the species studied certain variations exist in the relative abundance and arrangement of epithelial and subepithelial nerves The muscularis has a uniform cholinergic and a variable adrenergic innervation in different species

The intrinsic innervation of the mammalian urinary bladder including that of man is still unsettled Extensive studies on bladder function in different animals after selective stimulation of vesical nerves partial or complete denervation and administration of various autonomic dru s have led to the following contradictory conceptions

(1) The bladder musculature in its entirety is supplied by purely motor fibers from both sympathetic and parasympathetic nerves (Budge 1864) (2) Both sympathetic and parasympathetic nerves supply the entire bladder musculature the former are motor to the trigone and inhibitory to the rest of the bladder and the latter perform a reciprocal function (Zeiss] 1892 1893 1894 01 Elliott 0 Learmonth 31) (3) The detrusor muscle is supplied exclusively by parasympathetic and the trigone exclusively by sympathetic nerves (Rehfisch 1897 Fag e 02 Langworthy and Murphy 39) (4) The detrusor muscle is antagonistically supplied by both parasympathetic and sympathetic nerves and the trigone only by the latter (Kuntz and Succomano 44)

Morphologic data on the distribution of intrinsic vesical nerves have been based entirely on material stained by supravital methylene blue or silver impregnation techniques which are neither fully reliable nor specific Recently developed histochemical techniques for the selective demonstration of cholinergic (acetylcholinesterase-containing) and adrenergic (norepinephrine containing) nerves have been used in the present work to define specifically the distribution of these two types of autonomic nerves in the bladder of the cat dog rabbit and rat

## MATERIAL AND METHODS

The urinary bladders studied were obtained under light nembutal anesthesia (in

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travenous or intraperitoneal) from 49 adults animals eight male and three female cats four male and ten female dogs fifteen male and five female rats and four male rabbits In the rat the bladder was removed in toto in the other animals it was sectioned transversely at 3 to 6 levels or bisected in the median sagittal plane The specimens were quick frozen with dry ice acetone and stored in a sub zero refrigerator at − 80°C Two to 14 μ sections were cut at − 30°C with a Lipshaw cryostat Each specimen was so oriented that the entire transverse or sagittal diameter of the bladder was represented on the slide As many as 30 consecutive or alternate serial sections of the full bladder thickness were obtained from single blocks

*Demonstration of norepinephrine (NE)* The following modification of Falck s ( 62) technique was employed Mounted cryostat sections were rapidly transferred to a desiccator and dried over phosphorus pentoxide under vacuum for 25 to 45 minutes or dried at room temperature by a continuous current of cold air for a period of 5 to 15 minutes The dried slides were transferred to a Coplin jar containing 5 to 10 gm of trioxymethylene (paraformaldehyde) powder the jar was sealed and heated in an oven at + 80°C for 60 to 80 minutes At the end of this period the slides were dehydrated in absolute alcohol for 15 to 20 seconds passed in succession through a 1 1 absolute alcohol xylene mixture and two changes of xylene for 15 seconds each and mounted in Permount The dehydrating alcohol bath was replaced by 0 005% Eosin Y in absolute alcohol as a counterstain for some of the sections

The sections were examined with a Zeiss fluorescent microscope utilizing a high pressure mercury (Osram HBO 200) burner a BG 12 excitation filter and a 470 mμ barrier filter Under these conditions the NE containing nerves stand out prominently as finely beaded bright green fluorescent lines in marked contrast to the dull faint greenish yellow autofluorescent background

Black and white photographs were taken on 3000 ASA 4 × 5 Polaroid film (exposure time 8–120 seconds) Color photomicrographs were taken on Kodak high speed Ektachrome EHB 35 mm roll films (exposure time 10–40 seconds) the processing of the films was modified to give a film sensitivity equivalent to 400 ASA

*Demonstration of acetylcholinesterase (AChE)* The following modification of the technique described by Karnovsky ( 64) was used Mounted cryostat sections were dried at room temperature for 5 to 10 minutes and then fixed in 4% neutral formalin at − 4°C for 15 to 2[illegible] minutes The sections were rinsed in distilled water and incubated at 37°C for one and a half to three hours The incubation medium was prepared by dissolving 12[illegible] mg of the substrate acetylthiocholine iodide in 16 3 ml acetate buffer (15 8 ml of 0 06 N sodium acetate + 0 5 ml of 0 1 N acetic acid) and then adding the following solutions in the order given 1 2 ml of 0 1 N sodium citrate 2 5 ml of 30 mM cupric sulfate 0 5 ml of 4 mM tetraisopropylpyrophosphoramide (iso OMPA) 2 0 ml of distilled water and 2 5 ml of 5 mM potassium ferricyanide The pH ranged between 5 5 and 5 6 Following incubation the sections were rinsed in distilled water counterstained in Harris hematoxylin for 30 to 45 seconds blued in 1% lithium carbonate washed in tap water (1 minute) dehydrated in 95% and absolute alcohol (15 seconds each) cleared in 1 1 absolute alcohol xylene mixture and three changes of xylene (30 seconds each) and mounted in Permount Sites of AChE activity are marked by a finely granular reddish brown precipitate of cupric ferrocyanide

## RESULTS

### *Innervation of the cat bladder*

*A Subepithelial nerves*

Two freely interconnected groups of nerves are discernible in the lamina propria namely the deep and superficial subepithelial nerves

*Deep subepithelial nerves* In the deepest part of the lamina propria numerous sizable cholinergic (fig 1) and adrenergic nerve trunks and thin nerve bundles run in various directions circumferentially i e in a plane concentric with the epithelial lining Some of the nerves join ganglia (fig 2) or individual ganglion cells in the lamina propria Both types of nerves are

closely associated with deep subepithelial vessels as neurovascular bundles and give off three sets of branches

(a) *Vascular nerves* These accompany the deep subepithelial vessels chiefly the arterioles which like the related nerve trunks and bundles run in a circumferential plane The vascular nerves ramify around each vessel and arborize in the adventitia to form prominent and intricate cholinergic and adrenergic plexuses (figs 3 4) which send fine fibers into the outer part of the tunica media Occasionally the nerves also give off slender fiber groups or isolated nerve fibers which run independently in the lamina propria The vascular nerves and perivascular plexuses closely follow the deep subepithelial vessels their deep muscular branches which run in an outward direction between the innermost muscle bundles and their superficial subepithelial branches which proceed in a radial direction inwards i e perpendicular to and towards the epithelium (figs 5 6) Just before reaching the junction of the two layers of the lamina propria each vessel branches dichotomously to smaller channels which continue divergently into the superficial layer of the lamina propria In this layer the corresponding vascular nerves and perivascular networks become incorporated in the superficial subepithelial plexus

(b) *Radial subepithelial nerves* These are thin nerve bundles both cholinergic and adrenergic which are seen running through the deep layer of the lamina propria parallel to but independent of nearby vessels (fig 8) While most bundles ramify as a part of the superficial subepithelial plexus a few do not divide till they actually reach the epithelium Some isolated cholinergic and adrenergic fibers derived from the deep subepithelial nerves or their radial branches seem to end freely at various depths in the lamina propria (fig 7)

(c) *Muscular nerves* In many areas a deep subepithelial cholinergic or adrenergic nerve trunk or one or more of its branch bundles run into the inner muscularis (fig 1) where they ramify

Rich cholinergic and adrenergic perivascular networks in the deep layer of the lamina propria are present throughout the bladder However the complexity of arrangement of the deep subepithelial nerves is more striking in the base [*] than in the dome of the bladder commensurate with the more abundant vascular channels and nerve trunks or bundles that exist in the former region Large nerve trunks and sizable ganglia in the lamina propria are found only in the dome and upper part of the body of the bladder

*Superficial subepithelial nerves* These fibers are derived partly from the cholinergic and adrenergic radial subepithelial nerves and partly from the nerve bundles and networks associated with the radial supepithelial vessels Traversing the deep layer of the lamina propria the nerves follow distinctly tortuous courses with many U or S turns α loops or corkscrew like spirals In the dome and upper part of the body of the bladder the superficial subepithelial nerves are represented by a few scattered cholinergic and adrenergic nerves situated for the most part along the arterioles and some of the venules in the superficial layer of the lamina propria (fig 9) They have few branches and scarcely intercommunicate At more distal levels of the bladder the nerves progressively increase in number undergo more extensive ramification and inosculate more freely (fig 8) Consequently the nerves become more and more intricately arranged till at the bladder base they are interwoven into a highly complex network

The superficial subepithelial plexus is typically seen in the region corresponding to the trigone (figs 5 10) Radially directed adrenergic nerve fibers running in the deep layer of the lamina propria ramify and inosculate in the inner third of that layer to form a wide meshed network This pattern is seen in both transverse and sagittal sections of the bladder indicating a tridimensional arrangement of this plexus On the epithelial side the plexus sends a few branch fibers which traverse the superficial layer of the lamina propria and intercommunicate sporadically on their way to the epithelium

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[*] By "base" is meant that part of the bladder including the anterior and lateral as well as the posterior wall which lies distal to the level at which the ureters pierce the bladder wall on its posterior aspect The rest of the organ is referred to as the "body" and its uppermost part the "dome"

The main part of the cholinergic superficial subepithelial plexus is located within the narrow superficial layer of the lamina propria In both transverse and sagittal sections of the bladder two continuous layers of cholinergic nerves are seen one at the junction of the two layers of the lamina propria and the other immediately beneath the epithelium The deeper network receives its component fibers from the nerves running in the deep layer of the lamina propria and the more superficial one sends fine twigs to the epithelium The two networks are joined together by numerous freely ramifying nerve fibers which have a predominantly radial direction and form a narrow meshed plexus On the whole the cholinergic superficial plexus is more intricate than its adrenergic counterpart

B *Epithelial nerves*

The epithelial nerve fibers arise from the cholinergic and adrenergic superficial subepithelial plexuses as radial branches or continuations of their component fibers Besides a few thin nerve bundles — chiefly cholinergic — continue uninterruptedly from the deep subepithelial nerves through the whole thickness of the lamina propria without branching or communicating with other nerves on the way to end by breaking up into fine fibers upon reaching the epithelium Many epithelial fibers are associated with small epithelial blood vessels often apparently of capillary size

The innervation of the epithelium ranges from a meager supply by a few widely spaced fibers at the bladder dome to a rich arborization in the distal part of the bladder base

In the first part of their course the adrenergic epithelial fibers lie almost perpendicular to the epithelium They enter the basal epithelial layer as undivided fibers or after branching in a Y or T shaped manner (fig 11) In the latter case the branch fibers run divergently for a short distance in contact with the epithelium and bending sharply they penetrate the epithelium almost perpendicularly Inside the epithelium the fibers are identified only with difficulty being as a rule very thin and faintly fluorescent They follow straight or more commonly wavy courses and becoming more or less parallel to th surface they come to abrupt endings i the basal third of the epithelium

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C *Muscular nerves*

*General pattern* The cholinergic muscular nerves ramifying inside the r musculature arise as branches of inter muscular nerve bundles (fig 13) Most of these are derived from larger juxtamural nerve trunks which course in the connective tissue on the external surface of the organ Sizable nerves are always found between outer muscle bundles and progressively become smaller as they repeatedly subdivide into the depth of the muscle Some of the latter are derived from deep subepithelial bundles and some communicate with the deep subepithelial vascular nerves and perivascular network Most bundles show numerous interposed or superimposed nuclei

The cholinergic intermuscular nerve bundles follow a wavy course in the connective tissue separating large muscle bundles Together with their parent trunks they accompany interstitial and adventitial blood vessels collectively forming neurovascular bundles Vascular branches proceed towards the arteries along which they run for variable distances as individual fibers thin bundles or inter anastomosing fiber groups Ramifying to fine fibers in the adventitia of the vessel they form intricate intermuscular and juxtamural perivascular plexuses which save for the larger size of the vessels differ in no way from their subepithelial analogues already described Many muscular nerves are derived from the vascular nerves or perivascular plexus The nerve fibers related

to some of the moderate sized venules are in the form of loose and poorly developed networks

The adrenergic muscular nerves are de rived partly from nerve bundles and large nerve trunks composed of cholinergic and adrenergic nerve fibers (fig 14) and partly from purely adrenergic nerve bundles (fig 15) which are commonly associated with blood vessels They give rise to vascular branches which form a rich perivascular plexus (fig 16) and to thin fiber groups which run in the connective tissue between and inside muscle bundles Although the adrenergic perivascular plexus is uniformly rich throughout the musculature the adrenergic nerve bundles vary in number in different regions of the bladder They are more abundant in the base than in the body of the bladder and in the latter region between the outer than between the inner muscle bundles In the bladder base the adrenergic bundles are about equally abundant at all depths in the muscle layer but still are much less in number than the corresponding cholinergic nerves As a rule the adrenergic intermuscular nerves are equally distributed in the anterior posterior and lateral walls of the bladder

Many cholinergic nerves are connected with intermuscular and juxtamural gan glia of different sizes (fig 17) They are present throughout the bladder at all depths in the muscle layer but are larger and more abundant in the base especially between the outer muscle bundles and in the adventitial coat The ganglia are com posed of a variable number of cells of dif ferent shapes The majority of the cells show a heavy staining of their cytoplasm and appear dark brown all are related to and are usually encircled by fine cholin ergic fibers Some ganglia are connected with adrenergic bundles as well These ganglia enclose scattered moderately or faintly stained cells and cells which show a diffuse faint or bright fluorescence or a diffuse stippling with fluorescent granules In addition they constantly contain fine adrenergic fibers that run between or even surround individual ganglion cells (fig 18) Aside from the purely and predomi nantly cholinergic ganglia a few purely adrenergic ones composed of small clus ters of brightly fluorescent cells may be encountered in the depth of the muscle layer particularly in the bladder base

In most bladders examined one or more paraureteric cholinergic ganglia were con stantly encountered in the cleavage plane between the intravesical part of the ureter and the surrounding bladder musculature They were frequently connected with mod erately thick cholinergic nerve bundles that ran principally parallel to the ureter

*Terminal ramifications* Coursing for variable distances between the larger mus cle bundles the cholinergic intermuscular nerves divide and give rise to smaller branch bundles which follow the connec tive tissue between smaller muscle bun dles These in turn give still thinner fiber groups that lie between groups of muscle fibers Traced inside the latter the nerve bundles together with the muscular branches of vascular nerves lose their identity and their component fibers be come incorporated in an extremely intri cate nucleated plexiform network (fig 25) This "cholinergic neuroterminal plexus is composed of fine sharply defined strands showing a high AChE activity which run along and inosculate between and around individual muscle fibers Such is the com *plexity of this plexus that its extensive* ramifications virtually entangle each and every muscle cell in the bladder wall (figs 1 13 17)

On the whole the terminal ramifications of adrenergic muscular nerves have a far less intricate pattern than the cholinergic terminals The nerves apparently ramify dichotomously and their finer branches run divergently (fig 23) along and around smooth muscle cells Here and there the fibers communicate between or across the surface of contiguous muscle cells No-where in the bladder however is the pic ture of an extensively ramifying terminal plexus found Many terminal adrenergic muscular nerves take origin from the vas cular nerves or perivascular plexus lying in the lamina propria between muscle bundles or in the outer adventitial coat of the bladder

The distribution of terminal adrenergic fibers shows distinct regional differences In the bladder dome only few terminal fibers are found in relation to large groups of muscle fibers (fig 20) In the inner

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part of the muscle scarcely any fibers exist apart from the perivascular plexus (fig 19) At more distal levels in the body of the bladder the terminal fibers progressively become more abundant and are related to smaller and smaller groups of nerve fibers but still more fibers are present in the outer (fig 22) than in the inner (fig 21) muscle bundles There fore in the body of the bladder the rela tive abundance of adrenergic terminals follows two gradients the nerves in crease progressively in number as they are traced (1) at a given level from within outwards and (2) at the same depth in the muscle layer from the dome distally In the bladder base the termi nal adrenergic fibers are much more abun dant than in the body of the bladder each fiber being related to one or a few muscle fibers This picture is uniform throughout the depth of the muscularis in the base (figs 23 24) No differences in the in nervation pattern exist between the ante rior posterior and lateral walls of the bladder

*Bladder innervation in other animals*

A *Subepithelial nerves*

In the dog rabbit and rat bladder the subepithelial nerves generally have a much less complicated arrangement than those in the cat bladder though as in the latter they are considerably more abundant in the base than in the body of the bladder This applies to both cholinergic and ad renergic fibers In the rabbit and rat more nerves are consistently seen in the pos terior wall than in either the anterior or lateral walls

In the rabbit deep and superficial nerves can be discerned only in the base In this region the main arborization of the subepithelial nerves is represented by a single narrow meshed network that under lies the epithelium and lies in the circumferential plane Otherwise the ar rangement of the nerves resembles that seen in the cat bladder save for the dif ference in number and intricacy of ar rangement (figs 26 27)

In the dog the subepithelial nerves are conspicuously less abundant than in either cat or rabbit The superficial plexus in the bladder base lies at the junction of th two layers of the lamina propria Th epithelial branches of the plexus ru through the compact zone of the subm cosa with few connections, so that th picture is that of a very wide meshe network

In the rat definite subepithelial nerves whether individual fibers or perivascula plexuses cholinergic or adrenergic ar seen only in the base of the bladder, es pecially near the bladder outlet Even in this region they are sparse do not moscu late and include no definite nerve bun dles or ganglia Nowhere in the bladder are the fibers discernible as deep and superficial networks

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In the rabbit the innervation of the epithelium closely resembles that seen in the cat except that a far less number of nerves are seen in the former animal In the dog intraepithelial fibers can be iden tified only in the bladder base Even in this region they are widely separated and undergo very limited branching No plexi form basal network like that seen in the cat or to a less extent in the rabbit is found the fibers as a rule remaining indi vidually discernible to their termination In the rat neither cholinergic nor adren ergic epithelial fibers could be seen

C *Muscular nerves*

A uniformly rich cholinergic innerva tion of all parts of the bladder muscula ture including the trigone or the corre sponding area is found in all three animal species The distribution of cho linergic ganglia and nerves and the appearance of the neuroterminal plexus are similar to the cat bladder Paraure teric ganglia occur in all In the rat a stout nerve trunk is constantly seen pene trating the whole thickness of the poste rior bladder wall in a very oblique direc tion a short distance above the level of the bladder outlet

The adrenergic muscular innervation in the dog is very similar to that in the cat but in the rabbit and rat it presents some special features

Like in the cat the base of the rabbit bladder has the richest adrenergic mus

cular innervation In the musculature of the bladder body the relative abundance of the adrenergic nerves follows the same "gradients as in the cat bladder In both the base and body of the rabbit bladder a third gradient" is noted the muscular nerves increase progressively as they are traced from the anterior aspect of the bladder posteriorly Throughout the bladder the intermuscular and juxtamural perivascular networks around both arterioles and venules are exceptionally prominent No adrenergic ganglia were encountered in the rabbit bladder

In the rat the proximal third of the bladder is totally devoid of adrenergic muscular nerves and shows only poorly developed networks around the few arterioles in the adventitial and interstitial connective tissue that exist at that level In the middle third small groups of nerve fibers appear between the muscle bundles of the posterior and lateral walls of the bladder Their terminal ramifications are related to groups of muscle fibers and are about equally abundant throughout the muscle layer Meanwhile the perivascular plexuses become more prominent In the anterior wall very few nerves with limited branching are seen In the distal third of the bladder more intermuscular nerves more extensive terminal ramification and more complex perivascular networks are found The nerves are equally abundant at all depths in the muscle layer although they are still more prominent posteriorly and laterally The general pattern in the distal third of the bladder thus resembles that in the bladder base of other animals No adrenergic ganglia could be identified in the rat bladder A few juxtamural nerve bundles on the anterior and lateral aspects of the bladder are apparently continuous with nerve bundles or fibers in the adjacent connective tissue capsules of the corresponding lobes of the prostate

## DISCUSSION

The autonomic nerve fibers in the bladder wall are classified functionally according to their staining characteristics as cholinergic or adrenergic The adrenergic fibers whether they run individually as nerve bundles or in conjunction with cholinergic fibers in large nerve trunks are unequivocally postganglionic sympathetic fibers On the other hand the origin of cholinergic fibers cannot be ascertained actually they may represent preganglionic or postganglionic sympathetic or parasympathetic efferent fibers or afferent sensory fibers All preganglionic fibers whether sympathetic or parasympathetic in origin are cholinergic and are present only within large nerve trunks Since both sympathetic and parasympathetic ganglia are present in the bladder wall many of the larger nerve trunks in the adventitial coat muscularis and lamina propria undoubtedly contain preganglionic fibers Certain sympathetic postganglionic fibers namely the vasodilator (Burn 38) and sudomotor (Dale and Feldberg 34) are known to be cholinergic Whether or not sympathetic cholinergic vasodilator nerves exist in the urinary bladder remains unknown It has also been proposed by Burn and Rand (59 60) that the adrenergic postganglionic sympathetic fiber is in essence a cholinergic fiber at the termination of which the adrenergic transmitter norepinephrine (NE) is released That this hypothesis is not universally applicable is apparent from a variety of experimental histochemical and pharmacological data in the recent literature (Koelle and Valk 54 Koelle 55 62 65 Euler 56 Giacobini 57 Goodall and Kirschner 58 Muscholl and Vogt 58 Carlsson et al 64 Dahlstrom and Fuxe 64 Dahlstrom 65 Rice and Long 66) Our finding of adrenergic fibers intermingled with cholinergic fibers in large nerve trunks or occurring as nerve bundles which give rise to smaller fiber groups and individual fibers also offers no support to the Burn Rand hypothesis It may therefore be proposed that the majority of cholinergic nerve fibers in the bladder wall excluding those running in large nerve trunks are parasympathetic postganglionic fibers

The presence of autonomic ganglia in the mammalian bladder has been well documented Although it is generally conceded that the region near the entrance of the ureters has the richest supply of ganglia there is no agreement as to their relative distribution in the different layers of the bladder wall While Michailow

part of the muscle scarcely any fibers exist apart from the perivascular plexus (fig 19) At more distal levels in the body of the bladder the terminal fibers progressively become more abundant and are related to smaller and smaller groups of nerve fibers but still more fibers are present in the outer (fig 22) than in the inner (fig 21) muscle bundles There fore in the body of the bladder the rela tive abundance of adrenergic terminals follows two gradients the nerves in crease progressively in number as they are traced (1) at a given level from within outwards and (2) at the same depth in the muscle layer from the dome distally In the bladder base the termi nal adrenergic fibers are much more abun dant than in the body of the bladder each fiber being related to one or a few muscle fibers This picture is uniform throughout the depth of the muscularis in the base (figs 23 24) No differences in the in nervation pattern exist between the ante rior posterior and lateral walls of the bladder

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or regenerative functions of the bladder epithelium The majority of the intraepithelial nerve terminations closely resemble sensory nerve endings and may therefore subserve an afferent sensory function

While most of the subepithelial nerves are associated with blood vessels in the lamina propria some apparently run independently and end freely in simple branched or complex terminations similar to sensory nerve endings described in that layer Some nerve fibers within the muscularis also join specific end bodies which are apparently concerned with proprioceptive muscular sensation Like those in the epithelium these apparently sensory nerve fibers are much more abundant in the bladder base than in the dome These findings indicate that the sensory function is particularly *prominent in* — though by no means restricted to — the bladder base In relation to the epithelium this would be at variance with the view entertained by Schabadasch ( 34) that the afferent terminations in the mucosa are limited to the trigone

Up to the present the mammalian trigone — in conformity with its ontogenic and anatomical distinction (Kalischer 00) — has been believed to possess an innervation pattern which is distinctly different from that of the rest of the bladder (detrusor muscle) Although opinion still differs as to the innervation of the detrusor muscle it is unanimously agreed that the trigone is supplied exclusively by sympathetic nerves In this study it is shown that the trigone not only is richly supplied by both sympathetic and parasympathetic nerves but also has the same innervation pattern as the detrusor muscle on the anterior and lateral aspects of the bladder base In contrast to the parasympathetic innervation which is uniformly rich throughout the bladder the sympathetic supply shows important regional variations Though sympathetic nerves are equally abundant in all parts of the musculature of the bladder base their number varies significantly at various levels and at different depths of the musculature of the body of the bladder It is thus evident that if a regional functional differentiation of the bladder musculature is at all present on the basis of differences in innervation it is to be defined between the body and base of the bladder rather than between the detrusor muscle and trigone We would propose therefore that the two regions of the bladder namely the base and body differing in the type richness and pattern of innervation have distinctive motor as well as sensory functions

Whether or not all muscle cells in the bladder are supplied by nerve fibers has been a matter of contention The ratio of nerve fibers to muscle fibers has been variously estimated at 2–4 1 (Csiky) 1 1 (Obregia 1875 Gscheidlen 1877 Lustig 1881 Agababow 12) 1 25–50 (Englemann 1869) or 1 100 (Langley Stohr '28) Though a ratio of 1 100 is the one currently accepted (Bors 57) Caesar et al ( 57) *obtained evidence in an electron* microscopic study of the mouse bladder that "each and every muscle cell shows a close relationship to the axon at a well defined locus" The construction of the cholinergic neuroterminal plexus outlined in this report accords well with that of the peripheral autonomic innervation apparatus described in the literature (Stohr 32 Boeke 33 38 49 Hillarp 46 59 Jabonero 48 51 Myeling 53) and indicates that the terminal parasympathetic ramifications probably come in contact with every muscle cell in the bladder wall In view of its striking uniformity and continuity throughout the bladder musculature it is unlikely that the neuroterminal plexus would evoke a different response in different parts of the bladder musculature following one stimulus as advocated by the proponents of the reciprocal innervation theory Nor is it conceivable that the plexus would exert its influence on the muscularis on a strictly segmental or unilateral basis as emphasized in many previous reports (Giannuzzi 1863 Sherrington 1892 Griffiths 1894 Stewart 1899 Langworthy et al '34 Langworthy and Kolb '38 Jacobson 45 Carpenter and Root 51 Torbey and Leadbetter 63)

The sympathetic terminal fibers have an entirely different arrangement than the parasympathetic ramifications and are not associated with individual muscle cells Not even in the bladder base which

('08b), and Muller ('19) described ganglia in the submucosa as well as in other layers of the bladder wall, Bobin ('24) Hryntschak ('25) and Stohr ('28) admitted their presence only in the adventitia and muscularis Kuntz ('65) in a recent review asserted that an extensive area in the dome of the bladder is devoid of ganglia Our findings indicate that ganglia are present in all parts of the bladder and at all depths in its wall, including the lamina propria In conformity with previous reports the bladder base, especially at the entrance of the ureters and near the bladder outlet, contains the richest supply of ganglia Generally the deeper in the wall the ganglia lie, the smaller and fewer they are It is noteworthy that large ganglia and nerve trunks in the lamina propria occur only in the dome and upper part of the body of the bladder In the bladder base only thin nerve bundles and occasional isolated ganglion cells are present in the lamina propria

It was repeatedly pointed out that the intramural ganglia of the bladder are predominantly parasympathetic and partly sympathetic (Langley and Anderson, 1894 1896 Stewart, 1899 Kuntz and Moseley, '36 Moseley, '36) and not exclusively parasympathetic as maintained by some authors (Iljina and Lawrentjew, '32 Poly Karpowa, '35) In a study of the distribution of ganglia in the cat bladder Kuntz and Moseley ('36) stated that while parasympathetic ganglia — with few exceptions — are embedded in the deep portion of the serosa sympathetic ganglia are present in both serosa and external part of the muscularis In this study the ganglion cells in the bladder were found to contain AChE, NE or both In the cat and dog NE containing cells were encountered in all layers of the bladder wall, and in the body as well as the base of the bladder Though no NE-containing ganglion cells were encountered in the rabbit or rat bladder in this study, their presence cannot be entirely excluded until they are specifically looked for in serial sections of the whole bladder

The interpretation of the relationship of the different ganglion cell types to the parasympathetic and sympathetic systems has to be largely speculative Three types of ganglion cells are present in the bladder

*(a) Cholinergic cells* These are the most frequently observed They are AChE rich cells associated only with cholinergic fibers which ramify as postganglionic presumably parasympathetic fibers Therefore they are considered to be a part of the parasympathetic system

*(b) Adrenergic cells* These are quite rare contain only NE and show a diffuse bright fluorescence They give rise to adrenergic postganglionic sympathetic fibers and thus belong to the sympathetic system

*(c) Intermediate type cells* These are commonly found and are related to both cholinergic and adrenergic nerve fibers inside the ganglia While some stain heavily for AChE, others stain moderately or faintly for the enzyme and show as well a diffuse faint or bright fluorescence or a stippling with brightly fluorescent NE granules Whether this population of cells belongs entirely to either parasympathetic or sympathetic system or represent peripheral regulatory centers through which one system exerts a control over the other remains to be elucidated

Intraepithelial fibers have been depicted and described by a host of authors (Kisselew 1868 Retzius 1892 Grünstein '00 Lendorf '01 Michailow '08a Iljina and Lawrentjew '32 Schabadasch '34, Kleyntjens and Langworthy, '37, Langworthy and Murphy '39 Yokoyama '49, Yamaguchi '60 Seto '63) In this study it was found that the epithelium is supplied by both sympathetic and parasympathetic fibers which can be traced through approximately half of its thickness As a rule the adrenergic fibers are fewer and less complicated in arrangement than the cholinergic fibers However, both types of fibers show the same tendency to be more abundant in the base than in the dome of the bladder Many of the epithelial fibers are clearly associated with vascular channels Whether or not some fibers directly innervate the epithelial cells as well cannot be ascertained with the degree of microscopic resolution utilized in this study Should a cellular innervation exist it may prove to be related to the secretory

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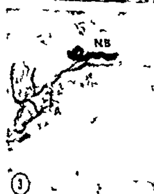
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MT
EP
NB
M
1


2


NB
A
3


A
4


5


E
6

All figures represent cat bladder except figures 14 and 18 (dog bladder) and figures 26 and 27 (rabbit bladder)

## PLATE 1

### EXPLANATION OF FIGURES

1 Deep part of lamina propria (LP) in upper part of body Large cholinergic deep subepithelial nerve trunks (NT) and smaller nerve bundles run circumferentially on the inner aspect of the muscularis (M) A nerve bundle (NB) courses into the inner muscularis The smooth muscle cells are surrounded by the extensive ramifications of cholinergic nerve fibers AChE × 40

2 Deep part of lamina propria in upper part of body Moderate sized cholinergic ganglion with adjoining nerve bundles AChE × 100

3 Deep part of lamina propria in base A cholinergic nerve bundle (NB) next to a deep subepithelial arteriole (A) sends vascular branches which form a plexus around the arteriole AChE × 100

4 Deep part of lamina propria in lower part of body Adrenergic perivascular plexus around arterioles (A) NE × 125

5 Superficial part of lamina propria in base cut tangentially The perivascular plexus around a radial subepithelial arteriole and its branches join the cholinergic superficial subepithelial plexus AChE × 40

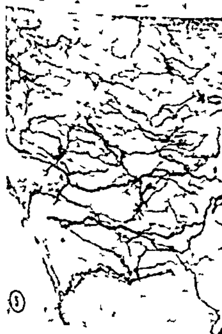
6 Epithelium (E) and lamina propria in lower part of body The adrenergic perivascular plexus around a radial subepithelial arteriole joins the adrenergic superficial subepithelial plexus NE × 80

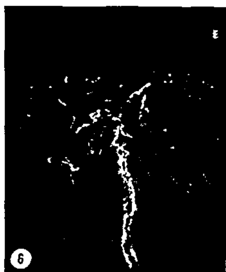

NT
LP
NB
M
1


2


NB
A
3


A
4


5


E
6

All figures represent cat bladder except figures 14 and 18 (dog bladder) and figures 26 and 27 (rabbit bladder)

## PLATE 1

### EXPLANATION OF FIGURES

1 Deep part of lamina propria (LP) in upper part of body Large cholinergic deep subepithelial nerve trunks (NT) and smaller nerve *bundles run circumferentially on the inner aspect of the muscularis* (M) A nerve bundle (NB) courses into the inner muscularis The smooth muscle cells are surrounded by the extensive ramifications of cholinergic nerve fibers AChE × 40

2 Deep part of lamina propria in upper part of body Moderate sized cholinergic ganglion with adjoining nerve bundles AChE × 100

3 Deep part of lamina propria in base A cholinergic nerve bundle (NB) next to a deep subepithelial arteriole (A) sends vascular branches which form a plexus around the arteriole AChE × 100

4 Deep part of lamina propria in lower part of body Adrenergic perivascular plexus around arterioles (A) NE × 125

5 Superficial part of lamina propria in base cut tangentially The perivascular plexus around a radial subepithelial arteriole and its branches join the cholinergic superficial subepithelial plexus AChE × 40

6 Epithelium (E) and lamina propria in lower part of body The adrenergic perivascular plexus around a radial subepithelial arteriole joins the adrenergic superficial subepithelial plexus NE × 80


7
8
NB


9


E
10


E


12

## PLATE 2

### EXPLANATION OF FIGURES

7 Deep part of lamina propria in lower part of body Free branche and complex terminations of cholinergic subepithelial fibers AChI × 100

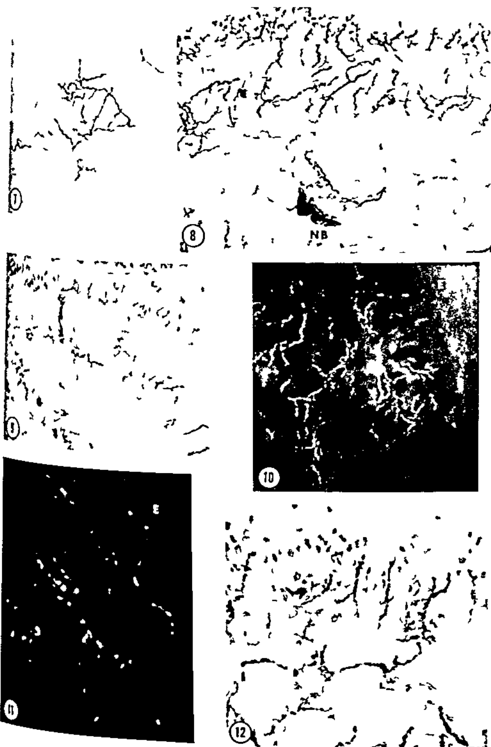
8 Epithelium and lamina propria in lower part of body Neurovascula bundle (NVB) in deep layer of lamina propria cholinergic superfici subepithelial plexus and epithelial fibers AChE × 40

9 Epithelium and lamina propria in dome In contrast to the ric cholinergic superficial subepithelial plexus and abundant epitheli fibers seen in the lower part of the body (fig 8) and the base (fi 5) only a few scattered cholinergic subepithelial fibers and n epithelial fibers are seen in the dome AChE × 40

10 Epithelium (E) and lamina propria in base cut tangentially Th adrenergic superficial subepithelial plexus is more prominent tha that in the body (fig 6) NE × 80

11 Epithelium (E) and superficial layer of lamina propria in base The epithelial branches of the adrenergic superficial subepithelia plexus run parallel to and immediately beneath the epithelium NE × 200

12 Epithelium and superficial layer of lamina propria in base Th cholinergic superficial subepithelial plexus gives rise to many epi thelial fibers which have free or branched clubbed terminations in it basal third AChE × 400


7
8
NB
9
10
E
11
12

## PLATE 3

### EXPLANATION OF FIGURES

13 Outer part of muscularis in lower part of body Large cholinergic intermuscular nerve trunks and neuroterminal plexus (cf figs 1 25) AChE × 40

14 Trigonal muscle Adrenergic nerve fibers in a large intermuscular nerve trunk NE × 320

15 Inner part of muscularis in lower part of body Moderate sized branching nerve bundle composed entirely of adrenergic nerve fibers NE × 125

16 Outer part of muscularis in lower part of body The adrenergic perivascular plexus around an intermuscular arteriole sends a branch fiber (BF) to adjacent muscle Adrenergic nerve fibers (NF) are seen inside a muscle bundle (lower right hand corner) NE × 125

17 Outer part of muscularis in base AChE rich ganglion cells in a large juxtamural ganglion A large cholinergic nerve trunk joins the ganglion AChE × 10

18 Outer part of muscularis in upper part of body Moderate sized intermuscular ganglion adrenergic fibers surround the ganglion cells some of which contain NE granules NE × 200


13


14


15


BF
NF
16


17


18

## PLATE 4

### EXPLANATION OF FIGURES

19 Inner part of muscularis in dome Adrenergic intermuscular perivascular plexus no muscular adrenergic terminal fibers NE × 125

20 Outer part of muscularis in dome Adrenergic intermuscular perivascular plexus few terminal fibers inside muscle bundles NE × 80

21 Inner part of muscularis in lower part of body Adrenergic intermuscular perivascular plexus more muscular nerve fibers than in the dome (figs 19 20) NE × 125

22 Outer part of muscularis in lower part of body More adrenergic muscular terminal fibers than in the inner part of muscularis at the same level (fig 21) NE × 80

23 Inner part of muscularis in base Adrenergic muscular innervation more than in the body (figs 21 22) Dichotomous branching and divergent course of terminal fibers NE × 125

24 Outer part of muscularis in base Same innervation pattern as inner part of muscularis at the same level (fig 23) NE × 80

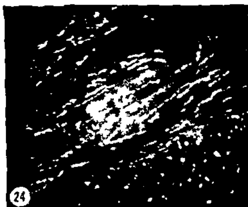

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## PLATE 5

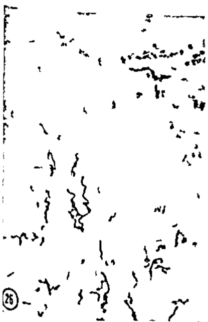
### EXPLANATION OF FIGURES

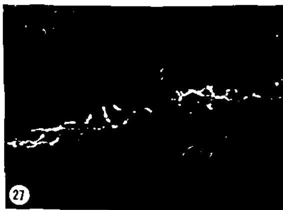
25 Muscularis in base Smooth muscle cells cut transversely and longitudinally are surrounded by a rich network of cholinergic fibers which are related to AChE-containing interstitial cells (neuroterminal plexus) A nerve bundle give rise to several branch fibers which are continuous with the plexus AChE × 160

26 Epithelium and lamina propria in base In contrast to the cat bladder (figs 5 8) the cholinergic subepithelial nerves are less numerous less frequently communicating and are mainly related to subepithelial vessels They send few fibers to the epithelium AChE × 40

27 Epithelium (top) and lamina propria in dome In contrast to he cat bladder (fig 9) only deep subepithelial perivascular adrenergic fibers are seen NE × 80


25


26


27

# Fine Structure of Adrenocortical Cells in Adult Male Rhesus Monkeys [1]

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ABSTRACT Adrenals of ten mature male rhesus monkeys were studied by electron microscopy

Mitochondria had lamelliform cristae in the zona glomerulosa and tubular cristae in the zona fasciculata and zona reticularis Agranular reticulum of tubular form was scarce in the zona glomerulosa but present in great abundance in the fasciculata and reticularis The Golgi complex was prominent in the cells of the zona glomerulosa but poorly developed in the cells of the inner cortical zones

Zona fasciculata cells contained large highly ordered "stacks" of granular endoplasmic reticulum which showed multiple connections with the random network of tubular agranular reticulum In the reticularis granular endoplasmic reticulum was present in more disordered whorl like arrays

Lipid droplets in the inner cortical regions were enveloped by tubules of agranular endoplasmic reticulum Occasional sections showed these tubules ending blindly at or near the surface of the lipid droplets This association was strongly suggestive of a functional relationship

Dense bodies varied in size and complexity of structure throughout the cortex They were smallest and simplest in form in the zona glomerulosa those in the zona reticularis were the largest and most complex

The adrenal cortex of the rhesus monkey (*Macaca mulatta*) has not been examined previously by electron microscopy Reports have been published on the electron microscopy of the following animals the frog (Burgos 59) the garter snake (Sheridan 63) the brown pelican (Sheridan et al. 63 Belt et al 66) the alligator and sea gull (Harrison 66) the domestic fowl (Fujita 61) the guinea pig (Sheridan and Belt 64) the opossum (Long and Jones 66) the rabbit (Cotte et al. 63) the hamster (Yates 65 Fawcett, '66) the mouse (Zelander 59) the rat (Lever 55 Sabatini and DeRobertis '61 Sabatini et al 62 Yamori et al 61 Giacomelli et al 65) and man (Ross et al. '58 Carr 61) Considerable species differences have been reported in mitochondrial structure the form of the agranular reticulum the amount and distribution of the granular reticulum the characteristics of the lipid droplets and the distribution of so-called dense bodies

The purpose of this report is to describe the fine structure of the adrenal cortex of adult male rhesus monkeys and to compare the findings with those in other species

## MATERIALS AND METHODS

Ten mature male monkeys ranging in weight from 8 to 14 kg were sacrificed according to a standard procedure Each autopsy was performed between 9 00 a m and 11 00 a m The animal was held immobile in a squeeze cage and Sernylan (Phencyclidine hydrochloride Parke Davis) was injected intramuscularly at a dose rate of 2 mgs per kg 5 to 15 minutes before the scheduled autopsy time The abdomen was opened the abdominal aorta was catheterized and 25–50 cc of blood were withdrawn for clinical test The adrenals were removed halved and placed directly into fixative the composition of which was 0 75% glutaraldehyde (prepared from Union Carbide 25% stock solution) 8% formalin (Merck analytical reagent grade) 1% sodium cacodylate 4 5% sucrose and 0 05% calcium chloride (the latter was added just before use)

The adrenals were fixed for 30 minutes and then sliced by hand into rectangular blocks representing complete radial sec

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tions of the cortex These slices were fixed for a total period of 24 hours Subsequently they were washed a minimum of 24 hours in two changes of a solution containing 4 5% sucrose and 1% sodium cacodylate Blocks were post fixed for two hours in a mixture of 1% osmium tetroxide 4 5% sucrose and 1% sodium cacodylate The osmicated tissues were rapidly dehydrated through an alcohol series to a 1 1 mixture of 70% alcohol and 70% acetone after which they were carried through an acetone series to a 1 1 mixture of acetone and Epon and then embedded in Epon (Luft 61) Sections with bright gold interference colors were double stained with uranyl acetate and lead citrate and examined in a Philips EM 200 electron microscope

The glutaraldehyde formaldehyde fixative listed above was devised after several trials in which the concentrations of each of the components were varied over a wide range In all such trials the criteria for optimal fixation were the minimization of shrinkage effects the preservation of intact mitochondrial structure the maintenance of homogeneous lipid droplet structure and the preservation of general tissue integrity Preliminary experiments were also carried out on the effect of the length of fixation time on general tissue integrity Tissues were fixed for 3 6 12 or 24 hours Cell preservation was best after fixation for 24 hours

## OBSERVATIONS

### *Capsule and zona glomerulosa*

The capsule of the adrenal gland is a multilayered feltwork of collagen fibers in which circular longitudinal and oblique layers of fibers alternate with one another in a fairly regular order The collagen bundles are in intimate contact with the arterioles and nerves which traverse the capsule before entering the cortex (fig 6)

Fibroblasts and macrophages are the only cellular components of the capsule The numerous fibroblasts are arranged in layers throughout the depth of the capsule (fig 1) The macrophages are fewer in number and are large cells containing several large electron dense granules a prominent Golgi complex and a considerable amount of agranular reticulum The c[ell] membrane of these macrophages is oft[en] extensively folded These cells are usua[lly] found either at the capsule glomerulo[sa] junction or in the subendothelial spa[ce] within the zona glomerulosa (fig 5)

The zona glomerulosa consists of shor[t] looped cords of cells which at the capsul[ar] end of the gland are separated from th[e] collagen fibrils of the capsule only by th[e] width of the narrow subendothelial spac[e] (figs 1 5 6) At the glomerulosa fascicu[lata] junction the glomerulosal cords eithe[r] merge with the outermost cells of the zon[a] fasciculata or form loops which abu[t] against the blood vessels of the fascicu[lata] forming a sharp transitional boun[dary] (figs 1 2)

The nuclei of the glomerulosa cells ma[y] vary widely in shape The majority ar[e] spherical (fig 6) but many are deeply lobulated (fig 10)

The mitochondria have lamelliform cristae which are plate like inward reflection[s] of the inner mitochondrial membrane (fig 8)

Small dense bodies are consistently found at the periphery of many glomerulosa cells Most of these bodies are round uniformly dense and bounded by a unit membrane but some are irregular in shape and have granular contents (figs 5 6 8) Their diameters range from 120 mμ to 420 mμ and they probably are lysosomes although their content of acid hydrolase has not yet been measured

The endoplasmic reticulum is predominantly of the granular type surprisingly most glomerulosa cells show no agranular reticulum at all In figures 5 6 7 and 8 large areas of cytoplasm are visible which are completely devoid of agranular endoplasmic reticulum Figure 9 shows some regions which do contain tubules of agranular endoplasmic reticulum in association with the cell membrane The much more prevalent granular endoplasmic reticulum is randomly arranged except for an occasional region where mitochondria and lipid droplets are closely bounded by curved cisternal profiles of the granular endoplasmic reticulum (fig 7)

Numerous ribosomes in addition to their presence on the membranes of the granular endoplasmic reticulum are scat

tered throughout the cytoplasm in the form of polysomes (fig 7)

The Golgi region is quite prominent in the cells of the glomerulosa Figure 9 illus trates the stacked cisternae and small ves icles characteristic of the Golgi complex in glomerulosa cells No large Golgi vacuoles are present

The cell membrane is highly convoluted and is bounded by a basal lamina com posed of a matted fibrillar material (figs 5 6) The glomerulosa cells adjoin the characteristic subendothelial space which in this zone contains numerous collagen fibrils (figs 5 6)

*The inner cortical zones*

On the basis of their cytological charac teristics the inner cortical zones in adult male rhesus monkeys can be divided into three subregions The first zone is the outermost portion of the zona fasciculata the cells of which contain the largest num ber of lipid droplets (figs 2 11) This zone the extent of which is quite variable merges imperceptibly with the inner re gion of the zona fasciculata whose cells possess smaller amounts of lipid (figs 3 12) The latter zone then merges with the zona reticularis which in this species is not a very highly reticular zone (fig 4) The cells of the reticularis abut against the blood vessels nerves and fibroblasts which surround the adrenal medulla (figs 4 24) As in many other species reticu laris cells may be found scattered among groups of medullary cells (fig 4) Only a few fibroblasts accompanied by very small amounts of collagen are present at the reticularis-medulla junction (fig 24)

In all of the cells of the inner cortical zones the mitochondria have tubular cris tae This is most clearly seen in figures 12 and 14 but details of mitochondrial struc ture are also visible in figures 13 17 18 23 and 24 There are no mitochondria with purely vesicular cristae in any region of the cortex Further there is no evidence of any centripetal gradation of mitochon dria throughout the inner cortical zones

All of the cells of the inner cortical zones have an extensive network of agran ular endoplasmic reticulum the tubules of which have a cross-sectional diameter of approximately 600 A Figure 12 is perhaps the best demonstration of this network but figures 13 14 17 18 and 19 also illus trate the tubular form and pervasive dis tribution of this organelle

In certain regions of the cytoplasm of the inner cortical zone cells the tubules of the agranular reticulum become con tinuous with discrete layered masses or stacks of granular reticulum (figs 16 17) In the cells of both the outer and in ner regions of the zona fasciculata the stacks are large and highly ordered con taining up to twenty layers of ribosome studded cisternae with a regular spacing between the cisternal plates of approxi mately 140 mμ (fig 17) In the zona retic ularis the stacks occur in more disordered whorl like patterns (figs 20 21 22) It is important to note that there is consider able variation in the size of these stacks both from animal to animal and from cell to cell

As in the zona glomerulosa those ribo somes not attached to the surface of the granular reticulum are scattered at ran dom in the cytoplasm in the form of poly somes (fig 13)

The lipid droplets in the inner fascicu lata and the zona reticularis are less nu merous and smaller than those in the upper fasciculata In occasional sections which graze the ends of small lipid drop lets groups of tubules of the agranular endoplasmic reticulum appear to end blindly against the surface of the droplet (fig 18) In other sections which transect droplets at or near their centers numer ous circular profiles of tubules appear in close apposition to the lipid droplet surface (fig 19) These images suggest that lipid droplets are surrounded by runs of tubules which envelop the droplet and end blindly at various points on its surface A complete analysis of the junctional relationships be- tween the lipid droplets and the tubules of the agranular endoplasmic reticulum must await serial sectioning

Most inner cortical zone cells contain dense bodies which are larger and more granular than those in the zona glomeru losa (fig 20) The granules within the dense bodies vary in both size and density and one or more of the larger granules often forms a bulge at the periphery of the dense body These large peripheral gran

ules are composed of a light, homogeneous matrix surrounded by a narrow layer of extremely electron dense material (figs 20 21 22) In the zona reticularis cells, where the dense bodies are of greater size and complexity than those in any other region, there are frequent mergers be tween dense bodies (fig 20) A unit mem brane can usually be seen around the fused bodies (fig 22) These mergers cre ate a new class of structures which shall be referred to here as complex dense bodies (figs 22 23 24) Their contents, which are extremely variable include sev eral sizes of granules with or without dense limiting membranes short runs of membranous material and a granular background matrix Presumably these complex dense bodies are the lipofuscin or lipofuscin like pigment granules seen with the light microscope in the adrenal cortex of a variety of species (Deane 62), no histochemical tests on these structures in the monkey have as yet been performed

There is a marked centripetal gradation in the size and complexity of the dense bodies throughout the inner cortical zones The smallest ones are found in the outer most zona fasciculata (approximately 0 5 μ in diameter) and the largest in the inner most cells of the zona reticularis (approx imately 1 5 μ to 2 0 μ in diameter) Dense bodies which are intermediate in size and complexity are found in the intermediate regions of the cortex

*The subendothelial space*

There is an ubiquitous subendothelial space in the adrenal cortex of the adult male rhesus monkey similar to that de scribed in other species The ramifications of this space are clearly seen in figure 3 a light micrograph of the inner zona fas ciculata Figures 11, 12 13 and 14 illus trate this space at much higher magnifica tions and also show the great number of microvilli which extend into it from the cortical cell surfaces This extreme multi plication of surface area by the microvil lous extension of the cell membrane is most marked in the inner regions of the zona fasciculata Note however that even in the fasciculata the degree of develop ment of the microvillous surface may be variable Figure 16 illustrates a portion of the inner fasciculata in which the cells show long regions of intimate contact interrupted only at intervals by small micro villous zones

Throughout the subendothelial space is a loosely dispersed, fibrillar material which also forms the basal laminae adjoining the surfaces of the cortical and endothelial cells (fig 13)

Collagen fibrils are found in considerable quantity in the subendothelial space particularly in the zona glomerulosa (figs 5 6) there are lesser amounts in the inner cortical zones

The endothelial cells are similar to those found in the adrenals of other species Their cytoplasm is highly attenuated and marked by the presence of characteristic annular pores These pores are capable of transporting relatively large masses of material Figure 15 shows a fragment of cytoplasmic material caught in the process of traversing an annular pore The source and motive power of the cytoplasmic fragment is not known how ever, pieces of cytoplasm may break away from the surface of a degenerating cell (of which there are always some in the cortex) wander within the subendothelial space and ultimately find their way into the blood stream *via* an annular pore This phenomenon is either rare or extremely rapid because it was observed only twice in this study

Macrophages frequently seen in the subendothelial space of the zona glomeru losa (fig 5), are not very common within the inner cortical zones

## DISCUSSION

*Mitochondria*

In the adrenal cortex of all species which have been described to date with the exception of the guinea pig and the opossum the mitochondria throughout the cortex have the tubular rather than the lamellar type of cristae In the guinea pig mitochondria with lamelliform cristae are found throughout the cortex (Sheridan and Belt 64) In the opossum (Long and Jones 66) mitochondrial cristae are lam melliform in the glomerulosa and tubular in the fasciculata The mitochondria in

the zona glomerulosa of the male rhesus monkey have lamelliform cristae while those in the inner cortical zones have cristae of the tubular type

Although there are as yet no published data on secretion of aldosterone by the adrenal cortex in the rhesus monkey we now have preliminary evidence that the adrenal of these animals secretes aldosterone *in vitro* (Kittinger 66) It is reasonable to assume that the zona glomerulosa is the source of aldosterone in the monkey as it is in the rat the cow and man (Stachenko and Giroud 62) Consequently we may infer that the presence of mitochondria with lamelliform cristae in the glomerulosal cells of the rhesus monkey is evidence that mitochondria with tubular cristae are not necessary components of steroid secreting cells The same conclusion must, of course be drawn from the presence of lamelliform mitochondrial cristae in the adrenal cortex of the guinea pig and the opossum

*Golgi complex*

The cells of the zona glomerulosa have a well developed Golgi complex in contrast to the cells of the inner cortical zones. However there is no evidence to permit even a reasonable speculation concerning the possible role of the Golgi in the cells of the zona glomerulosa

*Agranular endoplasmic reticulum*

There is considerable evidence (recently reviewed by Christensen 65) that the agranular reticulum in steroid secreting cells contains many of the enzymes essential to steroidogenesis Yet the cells of the zona glomerulosa of the rhesus monkey have very small amounts of agranular reticulum If aldosterone is really made in rhesus monkey glomerulosa cells as discussed above then the agranular reticulum may not be absolutely essential for aldosteronogenesis This however must be a tentative conclusion until we have more biochemical data on aldosteronogenesis in the rhesus monkey adrenal A similar tentative conclusion would also apply to the adrenal of the opossum Long and Jones (66) report that the cells of the zona glomerulosa of the opossum adrenal like those of the rhesus monkey have relatively small amounts of agranular reticulum

In contrast to the zona glomerulosa however the cells of the inner cortical zones are rich in agranular reticulum In the rhesus monkey this organelle always appears as a randomly disposed cytoplasmic network of interconnecting tubules whereas in the adrenals of most other species which have been described the agranular reticulum has been observed in the form of isolated vesicles

Fawcett (66) and Christensen (65) have pointed out the difficulties involved in preserving the delicate tubules of the agranular endoplasmic reticulum in the interstitial cells of the testis Christensen (65) has shown that the tubular form of the agranular reticulum in the interstitial cells of the guinea pig is only preserved when the testis is fixed by perfusion with glutaraldehyde fixation by immersion in glutaraldehyde always produces the vesicular form

In the adrenal cortex of the mouse (Brenner unpublished data) I have observed that immersion in glutaraldehyde or glutaraldehyde formaldehyde mixtures containing 4 5% sucrose always preserves the tubular form whereas the same fixative mixture without sucrose invariably results in the formation of vesicles

The tissues used in the present study were immersed in a glutaraldehyde formaldehyde fixative which contained 4 5% sucrose Immersion fixation was evidently adequate to protect the tubular system from breaking down into vesicles Perfusion fixation is therefore not essential for the preservation of the tubular form of the agranular endoplasmic reticulum *in the adrenal cortex It must be* remembered that species differences and organ differences in response to fixatives greatly complicate the task of finding a fixation technique which preserves all steroid secreting cells perfectly However the presence of the vesicular form of the agranular endoplasmic reticulum seems to be evidence of inappropriate fixation technique

*Granular endoplasmic reticulum*

The large and highly ordered stacks of granular endoplasmic reticulum present in

the inner cortical zones are undoubtedly of functional significance It seems reasonable to assume that high rates of protein synthesis occur within these stacks Since there is no cytological evidence of protein being made for export in adrenal cells the proteins manufactured in the stacks may be enzymes needed in adrenal cortical cell metabolism Since the stacks are in direct continuity with the tubules of the agranular endoplasmic reticulum where steroidogenesis is presumed to occur, the enzymes made may be involved in steroidogenesis The size of the stacks varies from cell to cell and from animal to animal Therefore variations in functional state may be reflected in variations in the amount of agranular reticulum converted by the cells into protein synthetic machinery This possibility is currently being investigated

In the guinea pig the adrenal contains a considerable amount of granular reticulum but Sheridan and Belt (64) did not describe stacks equivalent to those in the rhesus monkey adrenal Christensen (65) has observed junctions between the agranular reticulum and small stacks of granular reticulum in the interstitial cells of the guinea pig testis However the stacks were not prominent features of these cells and Christensen mentions that they occur in small patches (see his figs 3 4 12) Whether this structure is present in the adrenal cortices of other subhuman primates is currently under investigation Published electron micrographs of the adrenal of man do not show similar structures (Ross, 58 Carr 61) although Long and Jones (66) have reported that the human adrenal cortex does have similar large stacks of granular endoplasmic reticulum

### *Lipid droplets and the agranular endoplasmic reticulum*

It has been amply demonstrated with the light microscope that lipid droplets diminish in volume and often disappear completely during periods of maximal secretory activity of the adrenocortical cells When the secretory rates decrease the lipid droplets become larger This waxing and waning of the lipid droplets is presumed to correspond to the storage and release of steroid precursor molecu (Deane 51) The mechanism where the adrenocortical cell shuttles these pr cursors back and forth from lipid drople to steroidogenic sites remains obscu and it is not known whether or not the is an ultrastructural correlate for the fur tion of precursor transport

The areas of contact described abo (figs 18 19) between the tubules of th agranular reticulum and the surfaces the lipid droplets might be junctio across which steroid precursor transpo could occur If this were the case th frequency of appearance of these jun tions might vary directly with secreto rate This possibility is currently bein investigated

Two recent studies in other species vide evidence that the tubules of th agranular reticulum may become conflu ent with the surfaces of lipid droplets i organs other than the adrenal Geer (65 found lipid accumulations in small ci ternae of the agranular endoplasmic retic ulum in the foam cells and smooth muscl cells of fatty streaks in the human aort Smuckler et al (65) found stellate aggre gates of cisternae of both the granular an agranular endoplasmic reticulum su rounding and apparently leading into lipi droplets in the livers of guinea pigs intox cated with carbon tetrachloride

Although these are studies of patholog ical tissue they suggest that the tubule of the agranular reticulum have the poten tial to transport lipid

### *Effects of handling stress*

One must bear in mind that all of th observations in this study were made o the adrenals of animals which had under gone some stress because of handling True resting conditions in the adrenal ar very difficult to obtain short of sudde death of an unaware animal (Glick et al 54) Undoubtedly there were increases i ACTH blood levels in these animals befor they were sacrificed

Consequently the cytological observa tions reported here pertain not to restin cells but to normal cells mildly stimulate by endogenous ACTH In future reports shall compare the ultrastructural effect of intense exogenous ACTH stimulation

and the effects of severe cortisone suppres sion with the observations reported here Ultimately this should provide a complete picture of the full range of micromorphological variation which the adrenocortical cells of the monkey can express

ACKNOWLEDGMENT

I wish to express my thanks to Miss Mary Jane Kamm for her excellent tech nical assistance during this study

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*Abbreviations*

aer agranular endoplasmic reticulum
ap annular pore
bv blood vessel
C capsule
CDB complex dense body
cf cell fragment
cg chromaffin granule
cm cell membrane
co collagen
db dense body
ec endothelial cytoplasm
F fasciculata
f fibroblast
G glomerulosa
g golgi
gc golgi cisternae
ger granular endoplasmic reticulum
gr granule
gv golgi vesicles
J junctions
L lipid droplet
M macrophage
Med medulla
m mitochondria
mv microvilli
N nucleus
n nerve
P polysome
rbc red blood cell
SES subendothelial space
wbc white blood cell

## PLATE 1

### EXPLANATION OF FIGURES

1 Portion of the capsule glomerulosa and outer fasciculata The glomerulosal cords can be seen to merge with the fasciculata cords in lower right of micrograph Looped glomerulosal cords are evident at lower left of picture × 250

2 Glomerulosa fasciculata junction Lower end of a looped glomerulosal cord is evident in upper half of micrograph Note that outer most fasciculata cells are very rich in lipid × 1850

3 Inner fasciculata *Cells here are much less rich in lipid than those* in the outer fasciculata Note extensive ramifications of the subendothelial space × 760

4 Reticularis medulla junction Note the minimal degree of reticularization Some cortical cells are scattered about the medullary islands × 332


1


G
L
2


bv
R
SES
3


R
Med
4

## PLATE 2

### EXPLANATION OF FIGURES

5 Capsule glomerulosa junction A large macrophage is present at the capsule glomerulosa junction The arrow points to the highly convoluted cell membrane of a glomerulosa cell Dense bodies are present in the cytoplasm of a glomerulosa cell × 8 310

6 Capsule glomerulosa junction Collagen bundles nerve fibers and blood vessels typical of the capsule are shown here Note small dense bodies in glomerulosa cells × 4 680

5 M cm L L im Glomerulosa

6 Capsule bv SES co L Glomerulosa

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5
M
cm
L
m
Glomerulosa

6
Capsule
n
bv
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Glomerulosa

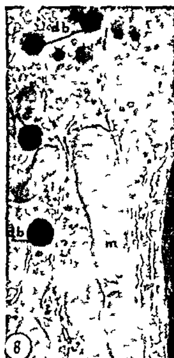
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6 Capsule-glomerulosa junction Collagen bundles nerve fibers and blood vessels typical of the capsule are shown here Note small dense bodies in glomerulosa cells × 4 680


L
ger
7


db
db
m
8




N
10

## PLATE 3

### EXPLANATION OF FIGURES

7 Glomerulosa cell The cytoplasm is characterized by mitochondria granular reticulum and polysomes Note lack of agranular reticulum × 18 500

8 Mitochondria in a glomerulosa cell Note lamellar cristae granular reticulum and small round dense bodies × 24 000

9 Golgi region in a glomerulosa cell Golgi cisternae and Golgi vesicles are visible × 16 500

10 Lobulated nucleus of a glomerulosa cell There is considerable variation in nuclear shape in the zona glomerulosa Most are round but many are deeply lobulated as illustrated here × 11 000


SSS
M
11

## PLATE 4

### EXPLANATION OF FIGURE

11 Outer fasciculata cell Cytoplasm is characterized by a large number of lipid droplets Note that the narrow subendothelial space is penetrated by microvilli A portion of a macrophage is visible at the lower right of the micrograph × 7400


11

## PLATE 5

### EXPLANATION OF FIGURE

12 Inner fasciculata cell The subendothelial space is penetrated by numerous microvilli The cytoplasm is characterized by mitochondria with tubular cristae agranular reticulum lipid droplets and dense bodies × 7 450 Inset shows the tubules of the agranular reticulum at a higher magnification × 37 600


ger
db
N
L
m
aer
db
12

## PLATE 6

### EXPLANATION OF FIGURES

13 Inner fasciculata This micrograph illustrates the thin layer of endothelial cell cytoplasm which separates the subendothelial space of the cortex from the blood stream Portions of an erythrocyte and a leucocyte are visible in the lumen Microvilli are present in the subendothelial space The cortical cells show an abundance of agranular endoplasmic reticulum and polysomes × 22 220

14 Inner fasciculata This micrograph illustrates the extensive intermingling of the microvilli from two cortical cells within the subendothelial space × 14 700

15 Inner fasciculata A cytoplasmic fragment (cf) bounded by a unit membrane (um) is shown during the process of traversing an annular pore (ap) in an endothelial cell (ec) × 62 500

SES
wbc
rbc
13






## PLATE 6

### EXPLANATION OF FIGURES

13 Inner fasciculata This micrograph illustrates the thin layer of endothelial cell cytoplasm which separates the subendothelial space of the cortex from the blood stream Portions of an erythrocyte and a leucocyte are visible in the lumen Microvilli are present in the subendothelial space The cortical cells show an abundance of agranular endoplasmic reticulum and polysomes × 22 220

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bv
CDB
m
20


DB
N
21


L
22


m
CDB
23

## PLATE 8

### EXPLANATION OF FIGURES

20 Reticularis Cells here are characterized by large and complex dense bodies Arrow points to fusion between dense bodies × 6 930

21 Reticularis An enlargement of part of figure 20 A single dense body Note the characteristic granule which is usually found at th periphery of the dense body The contents of the peripheral granule are more homogeneous than the rest of the body and the granule is always bounded by a very dense material × 21 900

22 Reticularis An enlargement of the body labeled CDB in figure 20 Formation of a complex dense body by fusion The arrow points to the membrane which surrounds both bodies × 21 000

23 Reticularis An enlargement of the body labeled CDB in figure 24 Complex dense bodies These are the largest and most complex dense bodies in the cortex × 24 700


L
DB
m
21


L
22


m
CDB
23

## PLATE 9

### EXPLANATION OF FIGURE

24 Reticularis medulla junction An unmyelinated nerve within the subendothelial space separates the medulla from the cortex The cytoplasm of a fibroblast is visible at the right The cortical cell cytoplasm is characterized by complex dense bodies presumably lipofuscin The medullary cytoplasm is characterized by chromaffin granules (cg) × 16 580


N
Reticularis
L
CDB
Nerve
SES
Medulla
cg
m
24

# A Dynamic and Static Study of Hepatic Arterioles and Hepatic Sphincters [1,2]

ROBERT S McCUSKEY [3]

*Department of Anatomy Western Reserve University Cleveland Ohio*

ABSTRACT The purpose of this investigation was to demonstrate the cytology of the sphincters of hepatic sinusoids to elucidate further the pathway by which arterial blood is distributed to the sinusoids and to study the hormonal and local regulation of arterial and portal venous blood flow through the sinusoids

The sphincters were found to consist of reticulo-endothelial cells and were the primary site for the regulation of blood flow through the sinusoids By contracting independently or in unison the flow of blood was regulated through individual sinusoids through sinusoids supplying a portion of a lobule or through sinusoids supplying a whole lobule When the sphincter cells contracted their nuclear region bulged into the lumen thereby occluding it

Hepatic arterioles were found to wind around adjacent portal venules with an average curvature of 42 and communicated with sinusoids via arterio-sinus twigs No structural arterio-portal anastomoses were observed however functional "arterio-portal anastomoses" were formed by short twigs which terminated in sinusoids near their origins No branches were found to terminate near central venules

The data suggest that the local regulation of blood flow through the hepatic sinusoids is mediated by vasodilator metabolites (adenosine and/or potassium) released from hypoxic hepatic cells as a result of the rapid glycogenolysis that accompanies hepatic cellular hypoxia

The liver consists of many identical morphological units that fulfill its numerous functions Each unit is microscopic in size and consists of a sinusoid with its afferent and efferent vascular connections and the surrounding hepatic tissue with its biliary lymphatic and nervous connections As liver function is dependent upon the conveyance of substances to and from these functional units a knowledge of the dynamic anatomic and physiologic relationships of the vascular system with each unit is required for an understanding of liver function

The current concept of the hepatic microvascular system is based primarily upon the investigation of the living liver of frogs by Knisely et al (48) and in mammals by Irwin and MacDonald (53) and Bloch ('55) Several structures seen in the living liver by these investigators however were seen infrequently were resolved incompletely or have not been seen by others As a result the following problems have been answered only partially (1) the reason for the closure of most of the hepatic arterioles during anesthesia is unknown (2) the cytology of the afferent and efferent sphincters of the sinusoids is almost unknown and (3) the local and hormonal regulation of blood flow through the sinusoids is understood poorly

The purpose of this investigation was to demonstrate in life the cytology of the afferent and efferent sphincters and to elucidate the local and hormonal factors that acted on these structures to regulate the flow of portal and arterial blood through the sinusoids

## METHODS

Frogs (*Rana pipiens*) white rats (Sprague-Dawley) mice (Swiss-Webster) and rabbits (New Zealand albino) were used

The frogs were fed with pieces of fresh rat liver and by injecting a sterile non pyrogenic 5% dextrose solution into the

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[2] Portions of thi rese rch were reported at the 13th Microcircul tory Conference Atl ntic City April 9 1965 and at the 78th Meeting of the American A sociation of Anatomi ts Miami Bea h April 21 1965

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subcutaneous lymphatic system, while the rats were fed a standard laboratory chow diet When starved animals were required food was withheld from frogs for one week and from rats 48 hours prior to their use

The frogs were anesthetized by injecting ethyl carbamate[4] (Urethane, 25%) into the subcutaneous lymphatic system and the mammals by an intravenous (tail vein) injection of sodium pentobarbital[5] (Nembutal, 10 mg/ml) or by an intraperitoneal injection of 25% ethyl carbamate Anesthesia was maintained by injecting 10% of the initial dose as required

The liver was exposed in frogs by making a paramedian thoraco-abdominal incision The mammalian liver was exposed by making a L-shaped abdominal incision The incision extended along the linea alba from just above the pubis to the xiphisternum and then continued laterally along the right subcostal margin to the tip of the twelfth rib A tracheotomy was performed and a tracheal cannula was inserted for the administration of oxygen (see below)

Movement of the liver that was induced by the respiratory system the heart and the gastrointestinal system was reduced by several methods Those caused by the respiratory system in mammals were reduced by cutting the falciform ligament to partially free the liver from the diaphragm Residual movements were eliminated by insufflating the lungs with oxygen introduced through a tracheal cannula using Irwin and MacDonald s (53) modification of the Meltzer and Auer technique or by paralyzing the diaphragm with an intravenous injection of succinylcholine chloride[6] (Anectine, 0 2 mg/ml) while administering intratracheal oxygen To reduce movements induced by the heart and gastrointestinal system the liver was immobilized on the mechanical stage of a modified compound binocular microscope This was accomplished by positioning a lobe of the liver over a bay window covered with Saran Wrap[7] and then immobilizing it with a movable clamp also covered with Saran Wrap This clamp gently held the liver without interfering with its microcirculation Gauze packing was placed between the liver and the intestines to eliminate the residual movements induced by the gastrointestinal tract

Livers which were immobilized on the stage of the modified compound binocular microscope were transilluminated with white light supplied by a 150 watt projection bulb or with light from an Osram 100 watt mercury vapor lamp that was projected through a Leitz prism monochromator and reflected from the substage mirror through the substage condensor Livers also were transilluminated with white or monochromatic light using the fused quartz rod technique of Knisely (36) as modified by Bloch (62)

The transilluminated liver was examined with a stereo-binocular microscope containing 4× 6× 8× or 12× objectives and 12 5× oculars a monocular or a compound binocular microscope containing 10× 22×, 50× 55× 75× or 90× water immersion objectives and 10× or 25× oculars

To obtain images of the liver on a television monitor a modified Dumont image orthicon television system was used (Bloch 63 64, 65) The television camera contained an image orthicon tube (RCA 5820) on to whose face plate the optical image of the liver was projected To secure maximum resolution (signal to image ratio) of a specific tissue component a magnification was selected which allowed the projected optical image to cover the largest possible area of the photocathode of the image orthicon tube (Bloch and McCuskey 66) Figure 1 is a block diagram of the optical and electronic system used

The hepatic microvascular system of each animal was observed an average of four hours with observations ranging from 30 minutes to 18 hours The hepatic microcirculation was considered healthy if the morphological criteria defined by Bloch (56) were fulfilled Animals that did not fulfill these criteria were not used

The dynamic events were recorded sequentially by the following methods (1) the images were observed microscopically measured with a calibrated ocular scale

[4] Ethyl carbamate (Urethane) Merck and Company Inc Rahway New Jersey
[5] Sodium pentobarbital (Nembutal) Abbott Laboratories North Chicago Illinois
[6] Succinylcholine chloride (Anectine) Burroughs Wellcome and Company Tuckahoe New York
[7] Saran Wrap Dow Chemical Company Midland Michigan

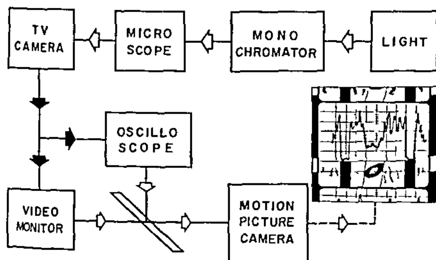



Fig 1 Block diagram of the optical and electronic equipment used for television microscopy of the living liver and scanning microspectrophotometry (from Bloch and Coyas 63)

and the information transferred to scale onto graph paper (2) photographs were made directly from the microscope with a 35 mm camera (Praktina FX) without a lens* and to produce additional magnification a 5× or 10× compensating ocular was used (3) photographs were taken with a 16 mm motion picture camera (Kodak Cine Special I) without a lens at framing rates of 8 to 32 frames per sec and a good image to frame ratio being produced by using the following projection oculars 1 6× 3 2× or 4 0× and (4) the images from the video monitor were photographed at a framing rate of 24 frames per second with a 16 mm camera (Maurer) having a f 1 6 50 mm lens or with the 35 mm camera (Praktina FX) equipped with a f 2 0 58 mm lens

The following pharmacodynamic substances salts and vital dyes were prepared and administered** Glucagon isopropyl norepinephrine epinephrine norepinephrine 5 ATP 5 ADP 5 AMP cyclic 3 5 AMP and adenosine were prepared in equimolar concentrations (1 μM/L to 100 mM/L) immediately before use Ionic salt solutions (NaCl Na SO KCl and K SO ) were prepared in equivalent solutions whose concentrations varied from 0 1 mEq/L to 100 mEq/L Glucose was administered as a 1% 5% 10% or 50% solution in isotonic saline Evans blue was injected as a buffered stock solution (0 4% ) and trypan blue was injected as a 1% or 2% buffered saline solution These solutions were administered in 0 1 ml doses by topical application to the liver surface with a micropipette or by intravascular injection

Dynamic blood pressures were recorded from portal and central venules of frogs using a modified Sanborn capacitance transducer system (Rappaport et al 59) A lobe of the liver was placed on a supporting frame which was covered with Saran Wrap Then a pyrex cannula with an orifice of 30 to 50 μ completely filled with degassed heparinized Ringer s solution and connected to the modified transducer system was positioned over the liver A portal or central venule was selected using the stereo-binocular microscope (at a magnification of 150×) and the cannula was inserted by means of a modified Chamber s micromanipulator (Bloch et al 61) The pressure pulses were dis

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* Kod chrom II Prof sional P natomic X, Plus X, Tri X film were used
P natomi X, Plu X, or Tri X film were used.
** These ubst nces were obt ined from th f llowing sources Glu gon and bet -d glucose Eli Lilly and Comp ny Indian polis Indi na 1 i propyl or epinephrin l-epinephrin nd l norepinephrine Winthrop Laboratories, New York, New York 5 ATP 5 ADP 5 AMP cyclic-3 5 AMP and adenosin Sig m Chemic l Company St. Louis, Mi souri Evan blue W rner-Chilcott, Morris Pl in New Jersey nd tryp n blu Allied Chemical and Dye Corpor tion, New York, New York.

played on an oscilloscope and recorded on thermosensitive paper with a Sanborn electrokymograph (Model 154-100B)

Serial sections were made of the livers that were observed *in vivo* The livers were fixed *in situ* by perfusion through the thoracic aorta with Zenker s fluid Helly s fluid, 10% neutral formalin, Carnoy s or Carnoy Lebrun fluid (Humason, 62) Then the liver was excised cut into 5 mm cubes and immersed in the fixative for 6 to 15 hours at 3°C After dehydrating in ethanol and clearing in benzene, the pieces of liver were embedded in Paraplast [11] and serial sections (3 μ–40 μ) made with a Minot microtome The sections were stained with one of the following stains hematoxylin and eosin Mallory s phosphotungstic acid hematin Casson s Heidenhain or Heidenhain s iron hematoxylin eosin orange G (Humason 62)

The sections were examined by light microscopy and compared with the histology observed in life The histology was recorded by camera lucida (Leitz) drawings and by 35 mm photomicrographs

## RESULTS

The transilluminated livers of 637 animals were examined (450 frogs 150 rats 25 mice and 12 rabbits) Qualitatively similar results were obtained from all species investigated

### *Hepatic arterioles*

Hepatic arterioles (and their branches) were seen in only 10% of the livers examined unless vasodilators were used to open them The ability to observe this small number of arterioles was influenced further by the animal s nutrition, its anesthesia the success in eliminating movements (induced by the heart respiratory and gastrointestinal system) and the method of transillumination Arterioles were seen more frequently in recently fed animals than in those which had been starved Furthermore they were visible more often in animals anesthetized with ethyl carbamate than in those anesthetized with sodium pentobarbital In animals lightly anesthetized with ethyl carbamate closure of hepatic arterioles was observed often when sodium pentobarbital was subsequently injected intravenously to maintain anesthesia. Closure of these vessels w observed often when oxygen insufflatio of the lungs was initiated to reduce mov ments induced by the respiratory systen but no changes in arteriolar blood flo occurred when these movements were sup pressed by immobilizing a lobe of the live on the stage of the modified compoun binocular microscope and the residual re piratory movements eliminated by par lyzing the diaphragm with succinylcholin chloride

Hepatic arterioles were difficult to ob serve with white light transilluminatio due to their small size and the light colo of the arterial blood which provided littl contrast with the surrounding tissue Ob servation of these vessels was facilitated however by transilluminating the liver with monochromatic light and using television microscopy which increased the contrast between its various tissue com ponents The following wavelengths light were found particularly useful 414 430 520 560 600 and 620 mμ contrast between the blood and its su rounding tissue was enhanced by wa lengths of blue light 407 414 and mμ permitting the patterns of blood to be observed easily Good contrast be tween vascular and parenchymal nents was obtained with wavelengths green light 520 540 and 560 mμ F thelium was visualized best when wave lengths of red light 600 and 620 mμ were used The best resolution of hepatic arteri oles was obtained when the modified com pound binocular microscope was used with 55× and 75× water immersion ob jectives

The spatial relationship of hepatic ar terioles with portal venules sinusoids and central venules was investigated These relationships were verified by injecting the dye Evans blue or trypan blue into the thoracic aorta which filled the hepatic arterial system prior to the filling of the portal venous system (fig 10) The relationships which were observed are illus trated diagrammatically in figure 2

Hepatic arterioles (and adjacent bile ducts) wound around adjacent distributing portal venules (portal venules from

[11] Paraplast Aloe Scientific St Louis Missouri.

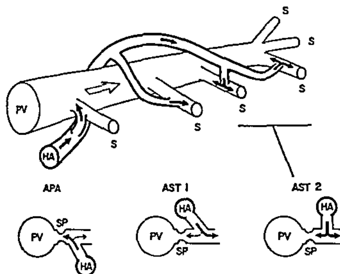



Fig 2 Schematic diagram of the relationships of the hepatic arteriole with its adjacent portal venule and sinusoids PV portal venule HA hepatic arteriole "APA" functional arterial portal anastomoses" AST arterio-sinus twigs S sinusoid SP inlet sphincter Solid arrows indicate direction of arterial blood flow Open arrows indicate direction of portal blood flow

which sinusoids originated) (figs 2 10–12) with an average curvature of 42 The internal diameters of these arterioles varied from 4 to 10 μ in the mammals to 7 to 30 μ in the frog and they were always smaller than the adjacent portal venules (see table 1)

Branches of the arterioles communicated with sinusoids via arterio-sinus twigs (AST's) These terminated in sinusoids at or near the periphery of the lobule None were found to terminate near the central venule Three types of AST's were observed (1) type 1 which terminated at acute angles to sinusoids directing arterial blood toward the central venule (figs 2 11 12) (2) type 2 which terminated at right angles to sinusoids (figs 2 11 12) and (3) short AST's which terminated near the origins of sinusoids from the portal venule (figs 2 11 12) All had an internal diameter equal to or less than a single red blood cell (3 to 10 μ for the mammals 7 to 25 μ for the frogs)

Direct connections between hepatic arterioles and portal venules were never seen in livers transilluminated with monochromatic light and examined at high magnifications (220× – 900×) with the modified compound binocular microscope Functional "arterio-portal anastomoses" ("APA s") were observed however and these were composed of two segments (1) a short arterio-sinus twig directed toward the portal venule and terminating in a sinusoid near its origin and (2) the short initial segment of the sinusoid with

TABLE 1

*Dimensional relationships of hepatic arterioles with adjacent portal venules*[1]

| Animal | P V | H A | A Curv | R.B C |
|---|---|---|---|---|
| | μ | μ | *degrees* | μ |
| Frog | 65 | 28 | | |
| | 50 | 28 | 42 | 15×25 |
| | 30 | 24 | | |
| Rat | 34 | 10 | | |
| | 28 | 10 | 52 | 6 |
| | 21 | 7 | | |
| Mouse | 30 | 9 | | |
| | 21 | 6 | 48 | 6 |
| | 15 | 6 | | |
| Rabbit | 50 | 10 | 26 | 6 |
| | 30 | 10 | | |

[1] P V., internal di meter of portal venule H.A., internal diameter of hepatic arteriole; Av Curv., average curvature of hepatic arteri le round d) cent portal venule (measurements taken from scale drawings nd individual motion picture frames u ing a protractor); R.B C., aver ge red blood cell dimensions.

its inlet sphincter which received the arterial blood (figs 2 11 12) Such APAs appeared to be direct connections between hepatic arterioles and portal venules however at low magnifications (100 – 150×) when white light quartz rod transillumination was used

The flow of arterial blood to the sinusoids was intermittent and lasted from a few seconds to several hours This was produced by arterioles, arterio portal anastomoses and arterio-sinus twigs which were independently contractile and opened and closed with no definable rhythm

Arterial blood flow modified the flow in portal venules sinusoids or both The various patterns of blood flow which were observed are illustrated in figures 2 11 and 12 When functional arterio-portal anastomoses ( APAs ) were wide open the arterial blood flow often reversed blood flow in the portal venule so that all sinusoids distal to the APA received only arterial blood (figs 2 11 12) If the APA" was dilated partially a mixture of arterial and portal blood reached the distal sinusoids (fig 11) Closure of the inlet sphincter eliminated the flow of arterial blood into the portal venule via the APA" and resulted in the sinusoid being perfused with arterial blood Under these conditions the APA functioned as an AST A sinusoid with an open inlet sphincter and an open AST was perfused with a mixture of arterial and portal venous blood (figs 2 11 12) With the inlet sphincter closed only arterial blood flowed through the sinusoid Arterial blood entering a sinusoid from an AST often was observed to flow into the portal venule through an open inlet sphincter especially if the outlet sphincter of that sinusoid was narrowed or closed and the sinusoid was filled with blood (figs 11, 12) Under these conditions ASTs of type 1 and type 2 often behaved functionally as APAs even though they terminated in sinusoids 20–30 μ away from a portal venule When blood flow in hepatic arterioles or in individual ASTs and APAs ceased portal venous blood often was observed to flow into AST's and APAs but rarely into hepatic arterioles Closure of the inlet sphincters eliminated this inflow of portal venous blood into APAs and AST's

In fixed preparations, the distribution of the hepatic arteriole and its terminal branches was similar to that observed *in vivo* Often a muscular sphincter was observed at the origins of the AST's from the hepatic arterioles, and although smooth muscle cells were seen occasionally in the more distal portion of these vessels none extended to the junction of the AST with the sinusoid

*Sphincters of hepatic sinusoids*

Good resolution of the sphincters of the hepatic sinusoids was obtained only with the modified compound binocular microscope and monochromatic light The best resolution of these structures was obtained at high magnifications (500–900×) with wavelengths of green light 520 540 and 560 mμ and with wavelengths of red light 600 and 620 mμ The lower wavelengths (green light) provided good contrast between the endothelial cells the hepatic parenchymal cells and the cellular components of the blood The higher wavelengths (red light) accentuated the endothelial cell membranes

The sphincters consisted of endothelial cells which were located at (1) the junctions of sinusoids with portal venules (inlet sphincters) (2) the junctions of sinusoids with central venules (outlet sphincters) and (3) the junctions of intersinusoidal sinusoids with sinusoids (sphincters of intersinusoidal sinusoids) (fig 3) At magnifications between 500 – 900× the nuclear regions of the sphincter cells were seen to enlarge frequently and to bulge into the lumen thereby occluding it (figs 4 5 16) The resulting reduction in internal diameter was accompanied by a thickening of the endothelial cytoplasm and a smaller reduction in the outside diameter of the sinusoid (figs 4 5) In many instances two or more endothelial cells opposed each other and were capable of reducing the lumen almost to zero either individually or mutually (fig 15)

Sphincter cells in all locations were similar morphologically The sphincter cells of dilated sinusoids had flattened ovoid nuclei with single prominent nucleoli and occasional refractile granules in their attenuated cytoplasm The nuclei of sphincter cells which were bulging and occlud

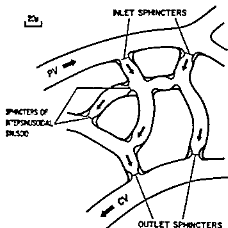



Fig 3 Semi schematic diagram of the location of hepatic sphincters PV portal venule CV central venule

ing the sinusoid lumen were more rounded and their nucleoli more distinct (fig 14) Such cells were demonstrated to be components of the hepatic reticulo-endothelial system (V Kupffer cells) since they phagocytized particles of trypan blue or India ink that were injected intravascularly Sphincter cells which were bulging and occluding the sinusoid lumen were still capable of removing foreign material from the blood stream Furthermore Kupffer cells containing phagocytized material continued to act as sphincters as demonstrated by their continued response to catecholamines

The remainder of the sinusoid appeared to be a smooth continuously lined tube whose internal diameter varied as changes in the internal diameter of its sphincters occurred A decrease in the internal diameter and a thickening of the endothelium usually accompanied the contraction of its sphincter cells

The sphincters regulated the flow of blood through the sinusoids by contracting independently or in unison As a result the flow of blood through individual sinusoids groups of sinusoids or the sinusoids supplying a whole lobule could be regulated independently of that in adjacent sinusoids groups of sinusoids or those of other lobules Open sphincters allowed rapid or moderate rates of blood flow through the sinusoid with no deformation of red or white blood cells When the sphincters were closed partially blood flow was slow and red blood cells squeezed through the narrowed sinusoid lumen (figs 4B and 14) Often this flow was impeded by less plastic white blood cells which stuck in the incompletely closed sphincters (fig 15) Complete closure of a sphincter prevented the flow of all cellular elements of the blood past that point (fig 16)

Concomitant with constriction of the inlet and outlet sphincters there was usually a change in the internal diameter of the

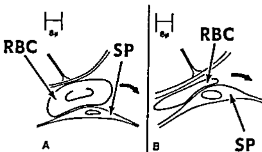



Fig 4 Outlet sphincters in the living frog liver Both A and B are tracings of single 16 mm motion picture frames photographed from the video monitor

A A non bulging outlet sphincter cell (SP) with red blood cell (RBC) passing through the sphincter

B A bulging outlet sphincter cell (SP) with red blood cell (RBC) being deformed as it passes through the sphincter

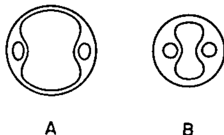



Fig 5 Diagrammatic representation of how sphincters constrict A is a cross-section of two relaxed non bulging sphincter cells B is a cross section of two contracted bulging sphincter cells Note the prominent bulging of the nuclear region the rounding of the nucleus and the thickening of the attenuated portion of the cytoplasm This results in a large reduction of the internal diameter of the sinusoid compared with a somewhat smaller reduction in its outside diameter

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The flow of arterial blood to the sinusoids was intermittent and lasted from a few seconds to several hours This was produced by arterioles arterio portal anastomoses and arterio sinus twigs which were independently contractile and opened and closed with no definable rhythm

Arterial blood flow modified the flow in portal venules sinusoids or both The various patterns of blood flow which were observed are illustrated in figures 2 11, and 12 When functional arterio-portal anastomoses ( APAs ) were wide open the arterial blood flow often reversed blood flow in the portal venule so that all sinusoids distal to the APA received only arterial blood (figs 2 11 12) If the APA was dilated partially a mixture of arterial and portal blood reached the distal sinusoids (fig 11) Closure of the inlet sphincter eliminated the flow of arterial blood into the portal venule via the APA and resulted in the sinusoid being perfused with arterial blood Under these conditions the APA functioned as an AST A sinusoid with an open inlet sphincter and an open AST was perfused with a mixture of arterial and portal venous blood (figs 2 11 12) With the inlet sphincter closed only arterial blood flowed through the sinusoid Arterial blood entering a sinusoid from an AST often was observed to flow into the portal venule through an open inlet sphincter especially if the outlet sphincter of that sinusoid was narrowed or closed and the sinusoid was filled with blood (figs 11 12) Under these conditions ASTs of type 1 and type 2 often behaved functionally as APAs even though they terminated in sinusoids 20–30 µ away from a portal venule When blood flow in hepatic arterioles or in individual ASTs and APAs ceased portal venous blood often was observed to flow into ASTs and APAs but rarely into hepatic arterioles Closure of the inlet sphincters eliminated this inflow of portal venous blood into APAs and ASTs

In fixed preparations, the of the hepatic arteriole and its branches was similar to that observed vivo Often a muscular sphincter was served at the origins of the ASTs from hepatic arterioles and although muscle cells were seen occasionally in more distal portion of these vessels extended to the junction of the AST the sinusoid

*Sphincters of hepatic sinusoids*

Good resolution of the sphincters of hepatic sinusoids was obtained only wi the modified compound binocular micro scope and monochromatic light The resolution of these structures was at high magnifications (500–900×) wi wavelengths of green light 520 540 560 mµ and with wavelengths of light 600 and 620 mµ The lower wave lengths (green light) provided good trast between the endothelial cells hepatic parenchymal cells and the lar components of the blood The wavelengths (red light) accentuated endothelial cell membranes

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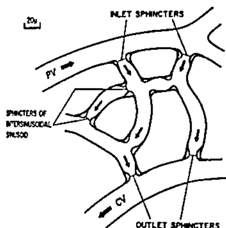



Fig 3 Semi schematic diagram of the loca tion of hepatic sphincters PV portal venule CV central venule

ing the sinusoid lumen were more rounded and their nucleoli more distinct (fig 14) Such cells were demonstrated to be com ponents of the hepatic reticulo-endothelial system (V Kupffer cells) since they pha gocytized particles of trypan blue or India ink that were injected intravascularly Sphincter cells which were bulging and occluding the sinusoid lumen were still capable of removing foreign material from the blood stream Furthermore Kupffer cells containing phagocytized material continued to act as sphincters as demon strated by their continued response to catecholamines

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The sphincters regulated the flow of blood through the sinusoids by contract ing independently or in unison As a result the flow of blood through individual sinus oids groups of sinusoids or the sinusoids supplying a whole lobule could be regu lated independently of that in adjacent sinusoids groups of sinusoids or those of other lobules Open sphincters allowed rapid or moderate rates of blood flow through the sinusoid with no deformation of red or white blood cells When the sphincters were closed partially blood flow was slow and red blood cells squeezed through the narrowed sinusoid lumen (figs 4B and 14) Often this flow was im peded by less plastic white blood cells which stuck in the incompletely closed sphincters (fig 15) Complete closure of a sphincter prevented the flow of all cellu lar elements of the blood past that point (fig 16)

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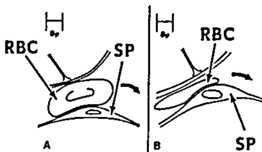



Fig 4 Outlet sphincters in the living frog liver Both A and B are tracings of single 16 mm motion picture frames photographed from the video monitor

A A non bulging outlet sphincter cell (SP) with red blood cell (RBC) passing through the sphincter

B A bulging outlet sphincter cell (SP) with red blood cell (RBC) being deformed as it passes through the sphincter

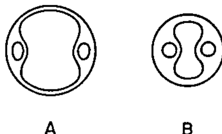



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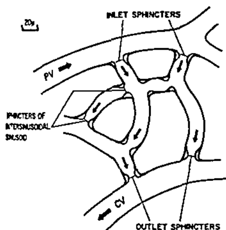



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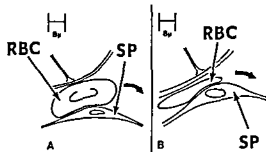



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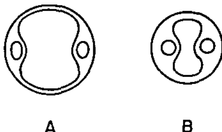



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remainder of the sinusoid lumen Such a *decrease in the internal* diameter was noted when the inlet sphincter alone constricted but this decrease usually did not reduce the sinusoid lumen to zero Sinusoids or segments of sinusoids which were devoid of blood cells appeared to be held open by the bulging nuclear regions of contracted sphincter cells located at the ends of intersinusoidal sinusoids Sinusoids were observed occasionally, however which contained no blood cells had little *lumen, and had a greatly reduced* outside diameter In such sinusoids no bulging endothelial cells were noted

The sphincters were responsible for the storage of blood within sinusoids or segments of sinusoids and for the release or autotransfusion (Knisely et al, 48, Bloch 55) of this stored blood into the general circulation via the central venules hepatic veins inferior vena cava and right heart Closure of the outlet sphincter alone caused the sinusoid to become distended with blood and with subsequent closure of the inlet sphincter this blood *was isolated from the general circulation* and stored In addition blood could be stored in isolated segments of sinusoids by the independent *action* of the sphincters at the ends of intersinusoidal sinusoids The blood stored in these intersinusoidal sinusoids could be released by the opening of their sphincters An autotransfusion reaction occurred when the outlet sphincter of a sinusoid storing blood opened and a peristaltic wave of contraction along the sinusoid wall milked the sinusoid of its contained blood into the central venule This reaction encompassed the many individual segments of a sinusoid and their respective sphincters

In histologic preparations the nuclear region of endothelial cells lining the sinusoids often bulged into the lumen at the afferent and efferent ends of the sinusoid and at bifurcations of the anastomosing sinusoid network (ends of intersinusoidal sinusoids) Pairs of these cells often were wrapped around the sinusoid lumen much like a collar (fig 17) Bulging endothelial cells had oval vesicular nuclei usually with single prominent nucleoli The cytoplasm was pale with a scant, dispersed basophilic granulation and occasionally contained phagocytized particulate material Non bulging endothelial cells had more compact, dense nuclei and nearly invisible cytoplasm In sections stained with iron hematoxylin, structures suggestive of fibrillar contractile elements were observed occasionally in the cytoplasm of the cells lining the sinusoid

*Effect of glycogenolytic hormones intermediaries, and metabolites on the hepatic microvascular system*

The hepatic microvascular system was affected significantly by glycogenolytic substances (glucagon, isopropyl norepinephrine epinephrine and norepinephrine), by adenine nucleotides (5 ATP, 5' ADP, cyclic 3', 5 AMP and 5' AMP) and by adenosine The effectiveness[11] of equimolar concentrations of the glycogenolytic substances in dilating intrahepatic blood vessels in decreasing order was glucagon ⩾ isopropyl norepinephrine > *epinephrine* > norepinephrine (fig 6) The effectiveness of equimolar concentrations of the adenine nucleotides and adenosine, in *decreasing order* was 5 ATP > 5 ADP > 5' AMP cyclic 3', 5' AMP and adenosine which were equivalent (fig 7) Other nucleotides or *nucleotide* derivatives pyrophosphate, and phosphate were ineffective vasodilators (McCuskey 63b)

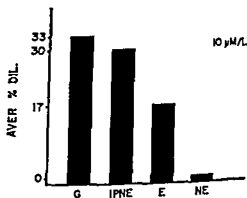



Fig 6 The effectiveness of equimolar concentrations of glycogenolytic substances in dilating intrahepatic blood vessels AVER % DIL average percentage of dilation of portal venules hepatic arterioles sinusoids central venules and sphincters produced by glucagon (G), isopropyl norepinephrine (IPNE) epinephrine (E) and norepinephrine (NE)

[11] Average percentage increase in internal diameter of intrahepatic blood vessels obtained after the administration of the test substances

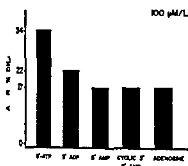



Fig. 7 The effectiveness of equimolar concentrations of adenine nucleotides and adenosine in dilating intrahepatic blood vessels AVER. % DIL, average percentage of dilatation of portal venules hepatic arterioles sinusoids central venules, and sphincters produced by adenosine-5-triphosphate (5 ATP) adenosine-5-diphosphate (5 ADP) adenosine-5 monophosphate (5 AMP) adenosine-3 5 phosphate (cyclic-3 5 AMP) and adenosine

Hepatic arterioles "APAs" and AST's were opened quite often by glucagon isopropyl norepinephrine and 5 ATP (tables 2 and 3) In addition these substances always caused dilation of portal venules sinusoids sphincters and central venules with a corresponding increase in hepatic blood flow Adenosine 5-diphosphate was slightly less effective than 5 ATP Cyclic 3' 5 AMP was a less effective vasodilator than glucagon isopropyl norepinephrine 5 ATP or 5 ADP Cyclic 3 5 AMP was as effective as 5 AMP adenosine or low concentrations of epinephrine

The response to epinephrine was dose dependent High concentrations (100 μM/L) caused constriction of portal venules hepatic arterioles central venules and sphincters and a drastic reduction in blood flow through the sinusoids with a concomitant autotransfusion" reaction The duration of this vasoconstriction and reduced blood flow was 10 to 30 minutes (Both the linear velocity and volume of blood flow were reduced ) Low concentrations of epinephrine (10 μM/L) however produced a biphasic response characterized by a brief phase of vasoconstriction (2 to 5 minutes) followed by a longer phase of vasodilation (10 to 30 minutes) with increased blood flow through the hepatic sinusoids In contrast norepinephrine generally produced effects similar to those observed with high concentrations of epinephrine but did not produce the biphasic response seen with low concentrations of epinephrine The vasoconstrictive effects of norepinephrine and high concentrations of epinephrine could be shortened by subsequent topical or intraportal administration of low concentrations of potassium as the chloride or sulfate salt isopropyl norepinephrine the adenine nucleotides and adenosine In contrast glucagon was effective only when applied topically to the liver surface

When potassium and sodium salts were parenterally injected or applied topically the following responses were observed Low concentrations of potassium (0 1 mEq/L) as the chloride (KCl) or sulfate ($K_2SO_4$) salt were dilators of portal venules sinusoids sphincters and central venules The hepatic arteriolar system was not affected significantly by these potassium salts Higher concentrations (10 mEq/L) of potassium salts however were vasoconstrictors of all vessels of the hepatic microvascular system No appreciable effect was observed when sodium as the chloride or sulfate salt was administered either topically or parenterally

TABLE 2

*Effect of glycogenolytic substances on the hepatic arteriolar system* [1]

| Drug | N | H.A. |
|---|---|---|
| | | % |
| Glucagon | 36 | 33 |
| Isopropyl norepinephrine | 33 | 30 |
| Epinephrine | 20 | 14 |
| Norepinephrine | 20 | 0 |
| Control | 244 | 10 |

[1] N number of experiments H.A. percentage of N in which hepatic arterioles opened following the administration of the drug

TABLE 3

*Effect of adenine nucleotides and adenosine on the hepatic arteriolar system* [1]

| Drug | N | H A |
|---|---|---|
| | | % |
| 5 ATP | 80 | 44 |
| 5 ADP | 10 | 25 |
| 5 AMP | 10 | 18 |
| Cyclic-3 ,5 AMP | 15 | 16 |
| Adenosine | 20 | 18 |
| Control | 244 | 10 |

N., number of experiments H.A percentage of N in which hepatic arterioles opened following the administration of the drug

Topical or parenteral beta d glucose (1%, 5%, and 10%) produced insignificant changes in intrahepatic blood vessel diameter or blood flow The highest concentration (50%) however reduced blood flow through the sinusoids and increased whole blood storage with little change in the internal diameters of portal and central venules

*Dynamic blood pressures in intrahepatic blood vessels*

Dynamic blood pressures from intrahepatic vessels were recorded only in frogs These pressures were recorded from portal and central venules whose diameter varied from 45 to 90 μ and the duration of these recordings was 10 to 30 minutes The range of blood pressures in these venules was considerable In 11 portal venules the pressures varied from 1 6/0 to 10 8/0 mm Hg while in a similar number of central venules the pressures varied from 1 9/0 to 10 3/0 mm Hg There was no correlation between their internal diameter and their dynamic blood pressures (table 4) However in three experiments the differences in dynamic blood pressures between a portal and central venule of the *same* lobule were secured The average pressure in these portal venules was 9 4/0 mm Hg while the average pressure in the central venules was 5 0/0 mm Hg indicating an average pressure drop of 3 8 mm Hg across the sinusoid bed in these animals (table 5) [13]

DISCUSSION

The results of this investigation have been fruitful in further elucidating (1) the terminal distribution of hepatic arterioles (2) the dynamic cytology of hepatic sphincters, and (3) the local and hormonal factors that act on these structures to regulate the flow of arterial and portal blood through the sinusoids The resultant concept of the dynamic anatomic and physiologic relationship of the hepatic microvascular system with the functional unit of the liver modified after Knisely et al (48) is illustrated in figure 8 and a theory for the metabolic regulation of blood flow through this unit is presented in figure 9 A discussion of these figures follows based on the results of this investigation

Examination of the hepatic arterioles in the living liver and those in fixed material generally reconfirmed the observations of Knisely et al (48), Irwin and MacDonald (53) and Bloch (55) that the mixing of arterial and portal blood occurred at or near the periphery of the lobule either in portal venules via arterio-portal anastomoses or in sinusoids via arterio sinus twigs or both In this study however the histology and distribution of these structures has been clarified further

Arterio sinus twigs (AST s) terminated in sinusoids near the periphery of the lobule in agreement with Kiernan (1833), Cameron and Mayes (30) Olds and Stafford (30) Wakim and Mann (42) Knisely et al (48) Senevirante (49) Andrews et al (49) Elias (49) Irwin

TABLE 4

*Dynamic blood pressures in intrahepatic vessels of the frog* [1]

| Portal venules | | Central venules | |
|---|---|---|---|
| P | I D | P | I D |
| | μ | | μ |
| 1 6/0 | 63 | 1 9/0 | 88 |
| 2 3/0 | 72 | 2 3/0 | 63 |
| 2 3/0 | 50 | 2 8/0 | 81 |
| 2 8/0 | 90 | 3 3/0 | 63 |
| 5 2/0 | 45 | 4 7/0 | 90 |
| 6 1/0 | 72 | 4 7/0 | 50 |
| 6 6/0 | 60 | 5 6/0 | 63 |
| 7 5/0 | 45 | 5 6/0 | 45 |
| 8 2/0 | 45 | 6 6/0 | 50 |
| 9 4/0 | 40 | 7 5/0 | 45 |
| 10 8/0 | 50 | 10 3/0 | 54 |

[1] P = blood pressure measured in millimeters of mercury I D = internal diameter measured in micra

TABLE 5

*Dynamic blood pressures in portal and central venules of the same lobule* [1]

| Portal venules | | | Central venules | |
|---|---|---|---|---|
| P | I D | | P | I D |
| 9 4/0 | 40 | | 6 6/0 | 50 |
| 8 2/0 | 45 | | 4 7/0 | 90 |
| 10 9/0 | 50 | | 5 6/0 | 45 |
| | | *Average* | | |
| 9 4/0 | 45 | | 5 6/0 | 62 |

[1] P = blood pressure measured in millimeters of mercury I D = internal diameter measured in micra

[13] The data on the effect of glycogenolytic substances intermediaries and metabolites on intrahepatic blood pressures were insufficient to report quantitatively

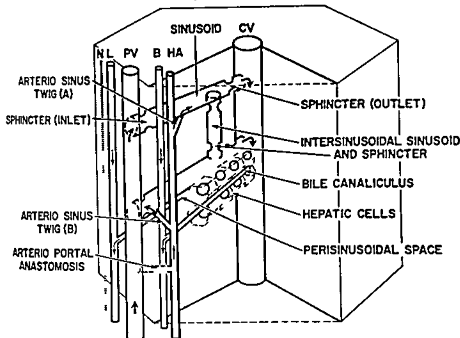



Fig 8 Schematic drawing of the functional unit of the liver and its relationship with the hepatic lobule (modified after Knisely et al 48) The functional unit of the liver consists of the sinusoid and its immediately adjacent hepatic tissue with their afferent and efferent vascular biliary lymphatic and nervous connections The anatomic components of this functional unit are (1) the sinusoid wall composed of reticulo-endothelial cells some of which function as sphincter cells and connective tissue elements (2) the hepatic cells among which are located the bile cannuliculi (3) the perisinusoidal space and (4) nerves Many functional units form an hepatic lobule PV portal venule HA hepatic arteriole N nerves L lymphatic B bile duct CV central venule

and MacDonald (53) Bloch (55) Riedel and Moravec (59) Lee et al (60) and Tajiri (60) However no twigs terminated in sinusoids near the central venules as described by Wakim and Mann (42) Senevirante (49) Elias (49) Elias and Petty (53) and Lee et al (60) and their existence is doubted seriously The translobular capillaries observed by these investigators may have been sinusoids with closed inlet sphincters which were conducting only arterial blood Such vessels fill with India ink or latex injected into the hepatic artery and appear as continuous vessels originating from hepatic arterioles

In this study the presence of functional arterio-portal anastomoses" ("APA s") in the periportal space was established These were short arterio-sinus twigs which were directed toward portal venules and terminated in sinusoids near their origins (from portal venules) forming part of the peribiliary plexus Although arterio-portal anastomoses similar to those reported by Wakim and Mann (42) Knisely et al (48) Senevirante (49) Irwin and MacDonald (53) and Bloch (55) were seen at low magnifications using white light transillumination such direct connections were not confirmed at higher magnifications when monochromatic light and television microscopy were used nor were they seen in histologic preparations While direct connections between the hepatic arteriole and the portal venule may exist they probably are rare

The distribution of arterial blood to the periphery of the lobule and the control of arterial blood flow through each AST and

'APA by independently contractile pre capillary sphincters permits critical con trol of the amount of arterial blood flowing to each functional unit or each lobule However, while the precapillary sphinc ters (*composed of smooth muscle cells*) regulate the flow of arterial blood *to* the sinusoids, endothelial sphincters in the sinusoids control the volume and rate of arterial and portal blood flow *through* each sinusoid Thus, *at any given moment each* sinusoid is capable of conducting only por tal blood only arterial blood or any mix ture of the two

In this study the sphincters that regulat ed blood flow through the sinusoids were found at the afferent (inlet sphincter) and efferent (outlet sphincter) orifices of *the sinusoids and in addition* at the ends of intersinusoidal sinusoids (sphincters of intersinusoidal sinusoids) The endothelial cells forming the inlet and outlet sphinc ters were synonymous with the afferent and efferent sphincter *mechanisms* de scribed by Knisely et al ( 48) Irwin and MacDonald ( 53), Bloch ('55) and *Knisely et al* ( 57) These sphincter cells were present in all observed species and failure to observe them by others (Sene virante 49 Maegraith 58 Kratochvel et al , 59 Brauer, 63) is attributed to poor *resolution due to the use of low magnifi* cations or poor definition due to excessive movements of the liver The demonstra tion of a significant *difference* of blood pressure between a portal venule and cen tral venule of the same lobule of the frog (table 5) and in the rat by Nakata Leong and Brauer ('60) is compatible not only with the *presence* of these sphincters but also with the concept that these sphincters are the primary site for the regulation of blood flow through *the sinusoids (espe* cially since there is little difference in blood pressure between mesenteric venules and portal venules (McCuskey, 63b))

All sphincters of hepatic sinusoids were similar *morphologically* and functionally All were contractile endothelial cells which by bulging were capable of re ducing the sinusoid lumen *to zero* or nearly zero and had the characteristics *of the* "gatekeeper" cells seen at bifurca tions of alveolar capillaries *in the living* lung (Bloch and McCuskey, 64) That endothelial cells might bulge into th sinusoid lumen and control blood flow ha been suggested *previously* by Ruttner Vogel ( 60) in their electron microscopi studies, but the present investigation i the first demonstration of their structu and function in the living liver at hig magnifications

The mechanism of endothelial tion however, is not clear but it may b *accomplished by contractile* fibrils structurally similar to myo- or fibrils In this study structures suggestiv of fibrils were seen occasionally and thes were similar to those reported in the out let sphincters of cevitamic acid treate guinea pigs (Warner 40) and in th reticulo-endothelial cells of the splee *(Leblond et al , 60) It should be noted* however that endothelial swelling migh be due to a change in intracellular osmo larity causing the cell to imbibe extra cellular water

*The sphincters* are extremely to vasoactive substances circulating in the blood or released locally and in response to these substances, rapidly modify the volume and rate of blood flow through the sinusoids While portal venules hepatic arterioles and central venules also are affected by these substances and can re*duce or increase* the amount of blood delivered to or removed from the sinusoids the sphincters are the structures respon sible for the regulation of blood flow through the sinusoids

The local and hormonal regulation of blood flow through the sinusoids is com plex and understood poorly Numerous *physiological and* biochemical data sug gest that glycogenolytic substances inter mediaries and metabolites may play a role *in the regulation of hepatic blood flow* Thus the regulation of blood flow through the sinusoids may be dependent upon gly cogenolytic events in the hepatic *cells In* vestigations of the effect of catechol amines and glucagon (potent glycogenolytic substances) on the hepatic blood vas cular system have shown that these ves *sels are affected significantly* by the administration of these substances (Bloch 40 Wakim 44 Senevirante 49 Daniel and Prichard 51 *Andrews et al* 55 56 Green et al , 59 Shoemaker et al 59

61 Blickman et al 60 Craig and Honig 63 Fischer 63 McCuskey 63a 65a b) Furthermore adenine nucleotides and adenosine which are intermediaries in glycogenolysis are also potent dilators of the hepatic microvascular system (McCuskey 63a, 65a b)

The reported effectiveness of the glycogenolytic substances (glucagon isopropyl norepinephrine epinephrine and norepinephrine) in stimulating glycogenolysis in decreasing order is glucagon ⩾ isopropylnorepinephrine > epinephrine > norepinephrine (Sutherland and Rall 60 Murad et al 62) This corresponds exactly with the observed effectiveness of these substances in dilating portal venules sinusoids sphincters and central venules (fig 6) and in opening hepatic arterioles (table 2) These results suggest that vasodilator metabolites or intermediaries might be released into the sinusoids from hepatic cells incident to glycogenolysis and that these substances might mediate the regulation of blood flow through the hepatic sinusoids

A theory for the production of vasodilator substances based on direct microscopic observation and the available biochemical data is presented in figure 9 It is postulated that adenosine and/or potassium released from hepatic cells during glycogenolysis or released as a result of hepatic cellular hypoxia induced by low blood $pO_2$ or increased hepatic cellular metabolism locally regulate blood flow through the sinusoids The precise biochemical mechanism by which adenosine and potassium mediate this regulation however is not understood

As indicated in the diagram (fig 9) glucagon isopropylnorepinephrine epinephrine and norepinephrine stimulate an increase in the enzyme adenyl cyclase which converts 5 ATP to cyclic 3 5 AMP (Sutherland and Rall 60 Sutherland

GLUCAGON ≥ ISOPROPYLNOREPINEPHRINE > EPINEPHRINE > NOREPINEPHRINE
ATP
ADENYL CYCLASE
CYCLIC 3 5 AMP
PHOSPHODIESTERASE
5' AMP
LIVER
DEPHOSPHOPHOSPHORYLASE
INACTIVE
KINASE SYSTEM
ATP
LIVER
PHOSPHORYLASE
ACTIVE
GLUCOSE
GLUCOSE 6 $PO_4$
GLUCOSE 1 $PO_4$
GLYCOGEN
VASODILATATION
$K^+$
INTRACELLULAR ADENINE NUCLEOTIDES
ADENOSINE
HEPATIC CELLULAR $pO_2$
HEPATIC CELLULAR METABOLISM
SINUSOID $pO_2$

Fig. 9 Theoretical scheme for the production of vasodilator metabolites in the liver

'APA by independently contractile pre capillary sphincters permits critical con trol of the amount of arterial blood flowing to each functional unit or each lobule However while the precapillary sphinc ters (composed of smooth muscle cells) regulate the flow of arterial blood *to* the sinusoids endothelial sphincters in the sinusoids control the volume and rate of arterial and portal blood flow *through* each sinusoid Thus, at any given moment each sinusoid is capable of conducting only por tal blood, only arterial blood or any mix ture of the two

In this study the sphincters that regulat ed blood flow through the sinusoids were found at the afferent (inlet sphincter) and efferent (outlet sphincter) orifices of the sinusoids and in addition at the ends of intersinusoidal sinusoids (sphincters of intersinusoidal sinusoids) The endothelial cells forming the inlet and outlet sphinc ters were synonymous with the afferent and efferent sphincter mechanisms de scribed by Knisely et al ( 48) Irwin and MacDonald ( 53), Bloch ( 55), and Knisely et al ( 57) These sphincter cells were present in all observed species and failure to observe them by others (Sene virante, 49, Maegraith '58 Kratochvil et al , 59, Brauer, 63) is attributed to poor resolution due to the use of low magnifi cations or poor definition due to excessive movements of the liver The demonstra tion of a significant difference of blood pressure between a portal venule and cen tral venule of the same lobule of the frog (table 5) and in the rat by Nakata Leong and Brauer ( 60) is compatible not only with the presence of these sphincters but also with the concept that these sphincters are the primary site for the regulation of blood flow through the sinusoids (espe cially since there is little difference in blood pressure between mesenteric venules and portal venules (McCuskey '63b))

All sphincters of hepatic sinusoids were similar morphologically and functionally All were contractile endothelial cells which by bulging were capable of re ducing the sinusoid lumen to zero or nearly zero and had the characteristics of the gatekeeper" cells seen at bifurca tions of alveolar capillaries in the living lung (Bloch and McCuskey, 64) That endothelial cells might bulge into sinusoid lumen and control blood flow been suggested previously by Ruttner Vogel ( 60) in their electron microsc studies, but the present investigatio the first demonstration of their struct and function in the living liver at h magnifications

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63) In contrast frequent closure of these vessels was observed when intra tracheal oxygen (100%) was adminis tered. It is obvious however that the hypothesis that blood flow through the sinusoids may be auto-regulated by oxygen lack requires further investigation under more controlled conditions (Ross et al 63 Garner et al 64) Rapid glycogenoly sis is known to occur during hypoxia but whether the dilatation of intrahepatic blood vessels and especially hepatic arteri oles during hepatic cellular hypoxia is me diated chemically by adenosine and/or potassium remains to be proven since the present evidence is circumstantial Obvi ously adenosine needs to be identified in the effluent blood of the liver during hy poxia, glycogenolysis and periods of in creased hepatic metabolic activity In ad dition the role of potassium needs to be evaluated further since it did not signifi cantly affect the hepatic arteriolar system which suggests that its role may not be important or may be secondary to that of adenosine

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Of interest i the recent identification of adeno ine in the efflu nt of the coron ry sinu in the isolated hypoxic heart (Berne 64b)

62) In brief the subsequent accumulation of cyclic 3′, 5-AMP activates a kinase to convert inactive phosphorylase to active phosphorylase resulting in the breakdown of glycogen to glucose in the hepatic cell Most of this glucose is released into the blood stream and can raise the level of blood sugar in the hepatic vein several hundred per cent (Ellis, 56 Craig, 58 Shoemaker et al, 59 Craig and Honig 63) Although glucose is released in large amounts it does not produce any significant change in the blood flow and thus does not appear to play a role in regulating blood flow through the hepatic sinusoids

The cyclic 3, 5′ AMP produced during glycogenolysis does not accumulate in large amounts since it is degraded continuously to 5′ AMP by phosphodiesterase (Sutherland and Rall, 60 Sutherland, 62, Butcher and Sutherland '62) Since the liver contains small concentrations of adenylic acid deaminase dephosphorylation of 5 AMP quite likely precedes deamination with the result that adenosine and not inosine is produced (Conway and Cooke 39) Adenosine and adenine nucleotides are potent dilators of intrahepatic vessels and sphincters (fig 7 table 3) as well as other vessels (Drury 36 Folkow 49 Winbury et al, 53 Duff et al 54 Furchgott 55, Wolf and Berne 56 McCuskey '63a '65a b) and therefore their role in the local regulation of blood flow through the sinusoids must be considered Of interest is the recent proposal of Berne ( 63 '64a) for the regulation of coronary blood flow Berne postulates that adenosine which is able to penetrate the cell membrane diffuses out of hypoxic myocardial cells and produces arteriolar dilatation This adenosine may arise from the intracellular breakdown of adenine nucleotides during hypoxia (Michal et al 59 Benson et al, 61) as a result of low blood p°, or an increase in myocardial metabolism A similar breakdown of adenine nucleotides has been observed in the hypoxic liver (Kayne et al '64) Although the adenine nucleotides (5 ATP 5′ ADP and 5 AMP) are potent vasodilators they are thought not to be involved since these charged nucleotides do not penetrate cell membranes (Lowy et al 52 58 Williams and Lepage, 58, Whittam, '6 Jacob and Berne 60 '61) That pare teral 5′ ATP and 5 ADP are more pote dilators than 5 AMP cyclic 3 5 AMP adenosine (fig 7, table 3) is presumed be due to the more rapid inactivation adenosine and AMP in the blood (Conw and Cooke 39)

Concomitant with glycogenolysis a lar efflux of potassium ion is observed in t hepatic venous blood prior to the elevati of the blood glucose concentration Th efflux of potassium is large enough double or even triple the normal seru potassium levels in hepatic venous bloc (Ellis 56 Shoemaker and Finder 6 Ellis and Beckett '63, Craig and Honi 63 Finder et al 64) Potassium is a p tent vasodilator of intrahepatic vessels an sphincters and is released along with gl cose in large quantities from the hypox liver (Ellis, 56 Cahill et al '57 Ellis an Beckett 63) This suggests that pota sium might be an important factor in th regulation of blood flow through the sinu oids especially since potassium also ha been demonstrated to cause vasodilatio in other vessels (Dawes 41 Scott et al 59 Waugh 62 Frohlich et al, 62) an a number of studies suggest that the mus cular tone of blood vessels and their sensi tivity to neural and hormonal stimuli ar determined partly by local changes in th concentrations of cations (Jang 40 Furchgott 55 Emmanuel et al 59 Scot et al 61 Frohlich et al 62 Hanenson 62 Bevan and Osher 64 Bohr 64)

Taken together the available data suggest that the regulation of blood flow through the sinusoids of anesthetized animals depends to a large extent upon the metabolic demands for oxygen by the hepatic parenchyma Hepatic cellular hypoxia resulting from increased metabolism or from lowered sinusoid blood p°, causes a concomitant opening of hepatic arterioles which increases the oxygenation of the blood perfusing the sinusoids Gross physiological studies of the changes in hepatic arterial and portal blood flow following the administration of glycogenolytic substances and during hypoxia indicate similar increases in arterial blood flow (Grabner et al 58, Cohn and Kountz 63 Fischer 63 Craig and Honig

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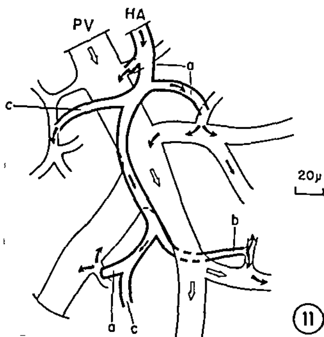
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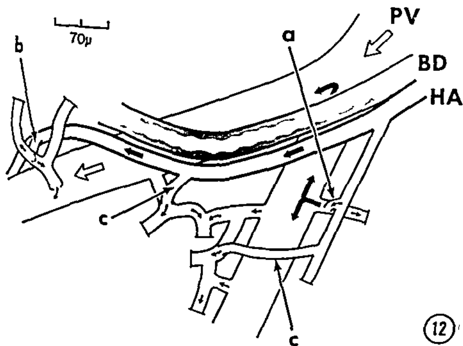
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HA
PV
10


PV
HA
a
c
20µ
b
a
c
11


70µ
b
a
PV
BD
HA
c
c
12

## PLATE 1

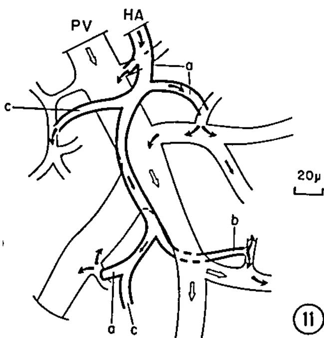
### EXPLANATION OF FIGURES

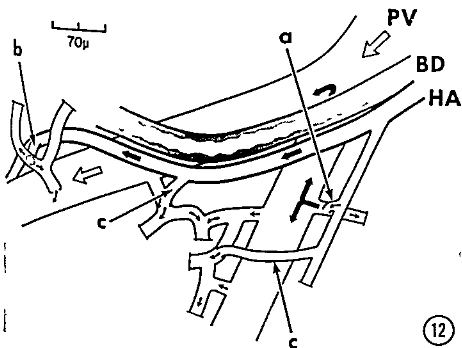
10 An hepatic arteriole (HA) and an arterio sinus twig (AST) selectively filled with Evans blue dye injected into the arterial system and adjacent to a portal venule (PV) Photographed from the living frog liver transilluminated with the white light using the quartz rod

11 Relationship of an hepatic arteriole (HA) with its adjacent portal venule (PV) and sinusoids (a arterio portal anastomosis b arterio sinus twig type 2 c arterio sinus twig type 1) Drawn to scale from the liver of a living rat transilluminated with monochromatic light (560 m$\mu$)

12 Relationship of an hepatic arteriole (HA) and bile duct (BD) with their adjacent portal venule (PV) and with the sinusoids (a arterio portal anastomosis b arterio sinus twig type 2 c arterio sinus twig type 1) Drawn to scale from the liver of living frog transilluminated with monochromatic light (560 m$\mu$)


HA
PV
10


PV
HA
a
c
20µ
b
a
c
11


70µ
PV
BD
HA
a
b
c
c
12

## PLATE 1

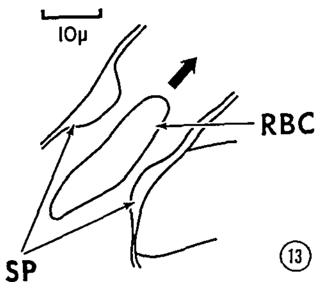
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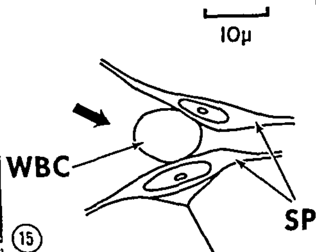
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10μ
RBC
SP
13


14


15


10μ
WBC
SP

## PLATE 2

### EXPLANATION OF FIGURES

13 Inlet sphincter (SP) in the living frog liver transilluminated with monochromatic light (414 mμ) The sphincter is dilated allowing red blood cells (RBC) to pass through it without deformation

14 Sphincter (SP) of an intersinusoidal sinusoid in the living frog liver transilluminated with monochromatic light (550 mμ) The sphincter is constricted partially and its nucleus and nucleolus (n) are prominent

15 Inlet sphincter (SP) in the living frog liver transilluminated with monochromatic light (560 mμ) A white blood cell (WBC) has stuck in the partially closed sphincter

A

B

C

10μ

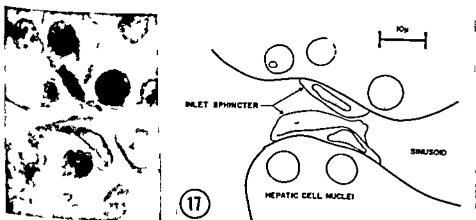
RBC

10μ

15μ

SP

(16)



## PLATE 3

### EXPLANATION OF FIGURES

16 Outlet sphincter (SP) in the living frog liver transilluminated with monochromatic light (560 mμ) The sphincter is closed impeding the flow of red blood cells (RBC) through it Only one cell of the sphincter was in focus A second cell on the opposite side of the sinusoid also was bulging into the lumen and the two cells working together completely blocked the flow of blood from the sinusoid into the central venule A B and C are frames from a motion picture of this sphincter These were taken to illustrate its sustained closure for 10 minutes (A) 20 minutes (B) and 30 minutes (C)

17 Histology of an inlet sphincter in the frog This is a composite camera lucida drawing of two 10 μ serial sections stained with hematoxylin and eosin one of which is illustrated in the photograph

# The Fine Structure of Ependymal Processes in the Teleost Optic Tectum [1]

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ABSTRACT Ependymal cell processes in the optic tectum of the adult sand bass *Paralabrax nebulifer* extend across the neural tube to the pial surface of the brain and exhibit several unique cytoplasmic features which readily distinguish these cells from other elements in the brain when examined in the electron microscope These include large perpendicularly oriented bundles of filaments cross striated by a series of irregular membranous structures ( ependymal reticulum") glycogen granules and dense-core vesicles in the outer portions and lateral protuberances of the main ependymal shaft, large clear vacuoles more numerous near the pial surface large mitochondria with a distinctive pattern of tubular cristae and a flocculent matrix and *fasciae occludentes* at sub-pial endfoot contacts These morphological findings suggest that ependymal cells do not simply constitute a primitive source for new cells or merely serve as structural support but rather are highly specialized and may display secretory activity

Early investigations of neuroglial elements primarily based on metallic impregnation methods in the brains of cyclostomes elasmobranchs and teleosts revealed long ependymal processes (Nansen 1886 Mirto 1895 Muller 00 Retzius 1893 Studnička 00 Cajal 11 Achucarro 15) In some regions of the brain in several lower vertebrate classes these long processes extend perpendicularly from the periventricular lining across the neural tube and expand into end feet below the pial surface This relationship of the ependymal processes (sometimes referred to as "tanycyte processes") to both surfaces of the neural tube appears to persist in adult specimens in some regions of the nervous system in most vertebrates Cajal ( 11) believed that the ependymal cells or "neuroepithelial" elements of most submammalian vertebrates constituted the sole non neuronal or neuroglial component of the brain parenchyma although his student Achucarro ( 15) recognized the existence of independent dendritic glial cells (*nevroglie autozome*) in some regions of teleost brains The presence of fibrillar material the extensive distribution of ependymal processes throughout the neuropil and the relative paucity of neuroglial elements in the teleost brain was interpreted by earlier workers as an indication that these cells are principally concerned with a supportive and nutritive function similar to that of mammalian neuroglia In addition many early workers (Achucarro 15 Agduhr 32) regarded the ependymal cell as a secretory or "glandular" element

The basal or perinuclear portion of ependymal cell has been studied recently in a number of vertebrates (Brightman and Palay 63 Oksche 58 Duncan 57 Bellairs 59 Schultz Berkowitz and Pease 56 Tennyson and Pappas 62 Klinkerfuss 64) but the fine structure of the long processes extending to the pial surface in lower vertebrate brains has not been described The present report is concerned with the fine structural characteristics of ependymal processes in the optic tectum of the adult marine teleost brain and especially with the description of some unique cytoplasmic features which distinguish these processes from all other nervous system elements

## MATERIAL AND METHOD

Material was obtained from seven teleosts (all Serranidae) of over 15 cms body length Animals were perfused by inserting a needle with flexible polyethylene tubing on the tip through the ventricle into the truncus arteriosus A variety of alde

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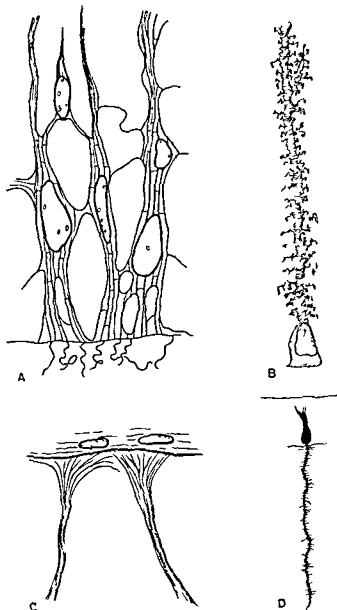



Fig. 1 Drawings of the ependymal processes of lower vertebrates seen with light microscopic techniques by earlier workers The ventricular surface is below and the pia above in all drawings

A. Iron hematoxylin preparation ow the arrangement of ependymal processes in *Petromyzon*. Note the orientation of processes and cross-striations (after Studnička 00)

B Methylene blue preparation of the basal ependyma ("neuroepithelial cells") and their processes in the frog (after Cajal 11)

C. Iron hematoxylin preparation of ependymal processes with their sub-pial end feet in *Scyllium* (after Studnička '00)

D Golgi preparation of ependymal processes with their sub-pial end-feet in the teleost (*Clupea sardina*) optic tectum (after Mirto 1895)

hyde fixatives and combinations with different buffers was studied and our most satisfactory material was obtained with a combination of 2% formaldehyde, 2% glutaraldehyde in a 0 1 molar cacodylate buffer and fish saline consisting of (in gm/1), NaCl 10 23, $CaCl_2$ 0 11, $MgCl_2$ 0 20 $NaHCO_3$, 0 024 and $NaH_2PO_4$, 0 002 The micrographs in this paper were all obtained from a single specimen of the sand bass, *Paralabrax nebulifer*, perfused with the above described mixture We are indebted to Sea World, San Diego and the Scripps Institution of Oceanography La Jolla California for providing fish and facilities

Immediately following perfusion the optic tectum was cut into strips of 1 mm or less extending to the ventricle (optocoele) These were rapidly transferred to cold cacodylate buffered 1% osmium tetroxide for 1 to 2 hours the tissue was rapidly dehydrated in ethanol and embedded in Araldite Thick plastic sections (0 5 1 μ) were cut on a Porter Blum microtome with glass knives and stained by several methods for examination in the light microscope Some thin sections were mounted on Formvar films over slotted grids for initial orientation but all of the illustrative material was obtained with thin unsupported sections Sections were stained with lead citrate and photographed in the Hitachi HU 11A electron microscope

## RESULTS

Ependymal processes in the optic tectum of adult teleost brains extend from the ventricular to the pial surface and constitute the majority of the extensively branched glial cells in metallic impregnation preparations The long processes which can be traced upward from each ependymal cell have been studied recently with various staining methods by Horstmann ( 54) and Fleischhauer ( 57) These elements have also been called neuroepithelial cells (Cajal '11) ependymal spongioblasts' (Penfield, 32) and somewhat similar elements in the developing mammalian neural tube have been identified in the electron microscope as "ependymal astroblasts" (Tennyson and Pappas 62, Klinkerfuss 64)

A striking feature of the long ependymal processes which persist in lower vertebrate brains is a series of grape like tuberosities (fig 1b) extending laterally along the course of the entire process (Cajal 11) The nuclei occur at varying depths but tend to concentrate at the ventricular surface (Studnička, 00 Agduhr, 32) At the surface the ependymal processes expand into broad end feet (fig 1) beneath the external limiting membrane (basal lamina) constituting a substantial portion of the brain surface (Studnička 00, Retzius 1893 Mirto 1895 Horstmann, '54 Fleischhauer, 57) Early observations with iron hematoxylin preparations (Studnička 00) revealed large longitudinal fibrillar bundles vacuoles and some cross striations (figs 1a c)

In the electron microscope profiles of the shaft of the ependymal processes rarely exceed 1–2 μ in diameter, but their distinctive features render them readily recognizable The central portion of each process contains a dense cytoplasm matrix filled with numerous fine filaments and surrounded by a less dense cytoplasmic periphery which is continuous with the lateral expansions As the processes approach the surface they expand as subpial end feet abutting with adjacent ependymal end feet or sub pial astrocyte processes The distinctive organelles of the ependymal processes include unique mitochondria longitudinally oriented fine filaments large clear cytoplasmic vacuoles dense core vesicles reminiscent of secretory granules or catecholamine containing vesicles glycogen and an unusual array of smooth endoplasmic reticulum

An electron micrograph of a typical ependymal process approaching the surface of the optic tectum is shown in figure 2 The cell can be distinguished readily from adjacent neuropil by the foamy appearance imparted by numerous large clear vacuoles of up to 0 6 μ in diameter These vacuoles display irregular contours and tend to be larger and more numerous near the pial extremity of the process where they are generally arranged in longitudinal rows At the surface, the process abuts the basal lamina and some vacuoles appear to fuse with the plasma membrane (fig 3 arrow) Another striking feature

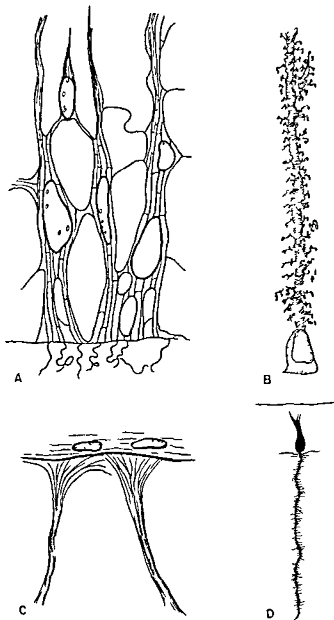



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C. Iron hematoxylin preparation of ependymal processes with their sub-pial end feet in *Scyllium* (after Studnička 00)
D Golgi preparation of ependymal processes with their sub-pial end feet in the teleost (*Clupea sardina*) optic tectum (after Mirto 1895)

is the presence of distinctive mitochondria of unusual configuration and large diameter predominantly oriented along the long axis in the outer portion of the process, and containing sparse irregularly disposed tubular cristae in a flocculent matrix of moderate electron opacity (figs 4, 5 6 11) With the present method of tissue preparation the cytoplasmic matrix density of the central region of the ependymal processes is usually distinctly greater than that of most of the surrounding neuropil

Numerous fine filaments of less than 100 A in diameter are aggregated in the central region of the process These bundles presumably correspond to the fine fibrils originally described by Muller ( 00) and Studnička ( 00) Similar filaments have been seen with the electron microscope in the perikaryal region of mammalian ependymal cells (Tennyson and Pappas 62, Brightman and Palay 63 Klinkerfuss 64) Following the ependymal process to a depth of approximately 50 μ from the surface the filaments are seen to be densely packed and oriented in the longitudinal axis of the main process with few if any extending into lateral branches (figs 6 11) Complex tortuous lateral expansions often can be traced for several microns and become interspersed between all elements of the neuropil Contained within these tuberous processes are large quantities of glycogen like granules large vacuoles and occasional accumulations of vesicles with an electron-opaque core (figs 2–13) but all of these organelles appear to be most concentrated in the sub pial end feet The small free granules of approximately 200–400 A display a marked electron opacity Because of their resemblance to glycogen in other tissues (Revel Napolitano and Fawcett 60) including astrocytes in mammalian brain (Maxwell and Kruger 65) we shall refer to these as glycogen although this has not been demonstrated experimentally in this tissue

The dense core vesicles consist of a cytoplasmic vesicle bounded by a single layered membrane, with a rounded central core matched only by glycogen in its electron opacity Many larger irregular vesicles are observed containing double or complex cores (figs 6–10) The range of vesicle size is 650 to 1600 A in outer diameter for vesicles with less complex cores Some profiles display small lateral membranous buds or retain attachment to vacuoles or to the flat tubular endoplasmic reticulum (figs 6–9) Although these dense-core vesicles are predominantly distributed within ependymal cells similar organelles usually of about the size of the smaller ependymal vesicles have been seen occasionally in axonal endings

A series of irregular membranous structures distributed in a plate like manner perpendicular to the long axis of the process appear in micrographs as cross striations with an irregular repeat period of approximately 0 2–0 35 μ (fig 11) These membranes are essentially planar tubular arrays displaying numerous anastamoses and a few lateral dilatations This tubular system might appropriately be designated the ependymal reticulum Specialized junctions or fusions of the plasma membrane similar to the fascia occludens described between ependymal cells lining the cerebral ventricle in mammals (Tennyson and Pappas 62 Brightman and Palay 63, Klinkerfuss 64 Brightman 65) have frequently been noted (figs 2 12 13) and similar junctional complexes can be seen to occur with astrocyte processes At these junctional regions between astrocyte or ependymal end feet the contours of the processes become complicated by irregular interdigitating processes united in extensive lengths of tight junctional complexes (fig 13) These tight junctions appear to be invariably present at the contacts of adjacent sub pial end foot expansions of ependymal cells but are rare or absent along the main shaft of the process The frequent association of tight junctions with electrical transmission (Robertson et al 63 Furshpan 64) may be indicative of a sub pial specialization for rapid ionic exchange in the teleost brain

## DISCUSSION

The ependymal cells of adult teleosts differ markedly from those of adult mammals (Tennyson and Pappas 62 Brightman and Palay 63 Klinkerfuss 64) in that they possess an unusually broad proc

es of specialized cytoplasm extending across the width of the neural tube The sub-pial expansions of these processes comprise a substantial portion of the surface area of the optic tectum The bundles of fine filaments or gliofibrils have been noted in the perikaryal region in mammals (Tennyson and Pappas 62 Brightman and Palay 63 Klinkerfuss 64) and reptiles (Fleischhauer 57) Although it is virtually impossible to trace a single ependymal cell through its entire course the width of the processes their orientation and their specialized organelles simplifies the identification of most well preserved portions of ependyma

The designation of the long ependymal cells that traverse the neural tube in fishes as supportive spongioblasts (Penfield 37) seems less than appropriate in the light of present-day knowledge It is also doubtful that these cells are transitional between primitive ependyma and astrocytes because of the more specialized nature of the cytoplasmic organelles in the tanycyte variety of ependyma

Ependymal cells display some features which are common to astrocytes These include the fine filaments and glycogen granules which are characteristic of all mammalian astrocytes (Maxwell and Kruger 65) In fishes (cyclostomes and teleosts) glial elements which have migrated from the ependymal layer possess these two organelles in quantities dependent upon their distribution and quality of preservation (Horstmann 54 Robertson Bodenheimer and Stage 63 Mugnaini and Walberg 65 Nakajima Bennett and Pappas 65 Kruger and Maxwell in press) and these cells would appear to be homologs of mammalian astrocytes The astrocyte may be *regarded* as the most direct derivative of spongioblasts which have migrated from the ependymal layer The ependymal cells which extend processes to the external limiting membrane of the brain might appropriately be called "ependymal astrocytes" (Tennyson and Pappas 62 Klinkerfuss 64) but these cells are clearly more specialized in terms of their component organelles than the astrocytes in the teleost central nervous system.

A supportive function for ependymal cells in submammalian forms was suggested on the basis of the broad relatively straight fibrillar processes and their orientation across the neural tube (e g Muller 00 Studnićka 00 Agduhr 32 Cajal 11) The ependymal reticulum orientated perpendicular to the longitudinal filaments may be related to the supportive function of ependymal processes but there is no direct evidence concerning their functional significance The striking resemblance of ependymal reticulum and filaments to the arrangement of myofilaments and sarcoplasmic reticulum is noteworthy although there is no evidence to suggest that these cells possess contractile properties

Numerous early workers (Studnićka 00 Cajal 11 Achucarro 15) also emphasized that ependyma play a glandular or secretory role in the brain as well as subserving special metabolic functions The choroid plexus epithelium may be regarded as a specialization of the ependyma and it is clear that its principal function involves secretory activity The contact of teleost ependymal cells with the ventricular and pial surfaces provides an unusual situation for exchange between the fluids bathing both surfaces Evidence of pinocytosis was observed in the ventricular portion of mammalian ependyma (Brightman 65) The extensive distribution of vacuoles in the sub pial processes may reflect transport in the direction of exchange of materials with the subarachnoid space

The extensive distribution of glycogen and the occasional contacts with capillary walls *en passage* as noted earlier by Achucarro ( 15) suggests that ependyma may parallel some of the functional properties of astrocytes which are the principal perivascular and glycogen laden elements in mammalian brain (Maxwell and Kruger 65) Distinct ependymal processes with vascular end feet have been demonstrated in fishes (Horstmann 54) amphibians (Bairati and Tripoli 54) and reptiles (Fleischhauer 57) It has been argued that gliovascular architecture is predominantly formed by ependymal processes in urodeles and anurans (Bairati and Tripoli 54) but electron microscopic evidence in

is the presence of distinctive mitochondria of unusual configuration and large diameter predominantly oriented along the long axis in the outer portion of the process, and containing sparse, irregularly disposed tubular cristae in a flocculent matrix of moderate electron opacity (figs 4 5 6 11) With the present method of tissue preparation the cytoplasmic matrix density of the central region of the ependymal processes is usually distinctly greater than that of most of the surrounding neuropil

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even more primitive vertebrates (e g cyclostomes) indicates that migrated neuroglial elements similar to mammalian astrocytes form most of the glial investment of capillaries (Mugnaini and Walberg 65)

The most remarkable morphological features suggestive of a specialized metabolic role for ependyma are the large quantities of glycogen and the mitochondria of unusual size and distinctive crista pattern Earlier suggestions of a glandular function (Achucarro, 15) based on the presence of secretory granules may find some support in the present study if the dense core vesicles could be interpreted as structural counterparts of secretory processes A variety of similar, but not identical granular or dense core vesicles have been noted in several portions of the nervous system especially in axonal endings The largest vesicles with dense cores range from less than 1000 Å to 4000 Å diameter and are generally referred to as neurosecretory vesicles or granules (Richardson, 62) Many but not all of the smaller granular vesicles of less than 1000 Å diameter appear to be related to catecholamines in axonal endings (Gray and Guillery 66) The presence of this broad class of vesicles with internal granular structure in axonal knobs in autonomic endings and in neurohypophysis has led to the interpretation that they are related to neurosecretory phenomena The dense core vesicles of somewhat similar morphology in non neuronal elements (i e ependymal cells) may be an expression of secretory phenomena unrelated to transmitter release at synapses The apparent origin of these vesicles from membranes of the smooth endoplasmic reticulum (most commonly seen in relation to the ependymal reticulum or the vacuoles that appear to arise from them) is consistent with the interpretation of these vesicles as a secretory product

The extensive Golgi apparatus in the basal portions (Horstmann 54 Fleischhauer 57, Brightman and Palay 63 Tennyson and Pappas 62 Klinkerfuss '64) and the ependymal reticulum would also appear to suggest that these elements might subserve significant synthetic function Pesetsky ('65) has demonstrated that thyroidectomy or thyroxin stimulation accompanied by alterations in size a number of ependymal cells as well as in number of histochemical changes in t developing anuran brain The recent demonstration of thyroid regulation of thi mine pyrophosphatase activity ( 66) suggests phasic activity in the Gol apparatus of ependymal cells The of distinctive organelles present in telec ependymal cells would appear to indica that these cells are not simple primiti supportive elements

ACKNOWLEDGMENTS

We are indebted to Mrs Olga Fiorell Mrs Sharon Sampogna and Dr Hor Schwassmann for assistance to Mr Krista Osterberg for the drawing and Mr Diana Brinton for typing the manuscrip

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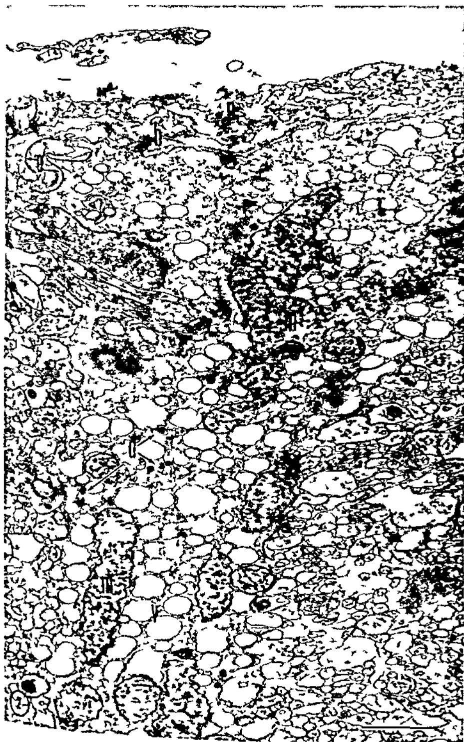


PLATE 1

EXPLANATION OF FIGURE

2 Electron micrograph of an ependymal process expanding into a subpial end foot Note the characteristic dense cytoplasmic matrix, large clear vacuoles (V) dense-core vesicles (d) and large mitochondria (m) with a flocculent matrix and tubular cristae Numerous glycogen granules can be seen in the ependymal cytoplasm beneath the basal lamina (b) separating the brain from the overlying pial processes (p) Near the surface an interdigitating process of an adjacent ependymal end foot forms a "tight junction" of the *fascia occludens* variety (o) Bar in this and all subsequent figures represents one micron


4


5

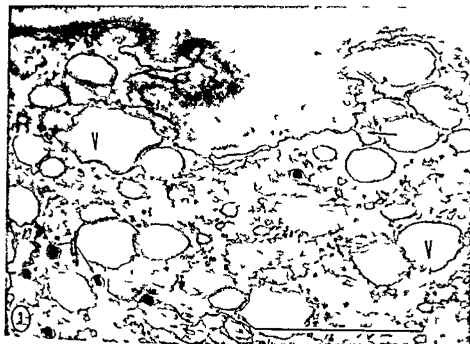
## PLATE 2

### EXPLANATION OF FIGURES

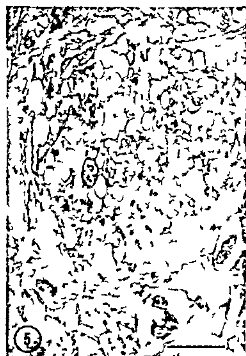
3 A sub pial ependymal end foot containing numerous large clear vacuoles (V) one of which opens onto the surface (arrow) Typical organelles of ependymal cytoplasm present include glycogen filaments and dense core vesicles (d)

4 A deep ependymal process cut perpendicular to its long axis The central core contains numerous filaments cut transversely and a cluster of membranes forming the ependymal reticulum The outer portions of the process contain mitochondria and large clear vacuoles

5 Transverse section of an ependymal process near the level of expansion to form a sub-pial end foot An increase in the number of large vacuoles and dense-core vesicles can be seen in the outermost (sub pial) portion of the ependymal process and they display a tendency to cluster into the lateral portion and side branches


V
V
1

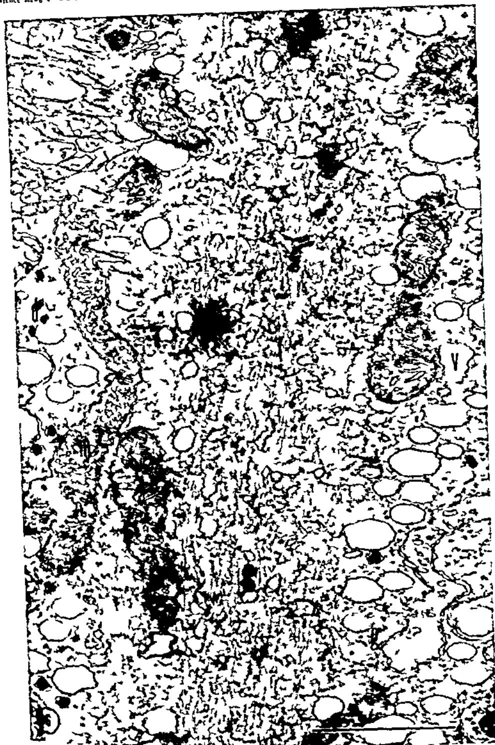

4


5

## PLATE 3

### EXPLANATION OF FIGURE

6 Ependymal process oriented perpendicular to the pial surface The central core contains large bundles of longitudinal filaments (f) irregularly striated by tubules or vesicles of the ependymal reticulum (er) The outer portions of the process contain large long mitochondria (m) clear vacuoles (V) glycogen granules (g) and dense core vesicles (d)

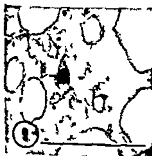


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## PLATE 4

### EXPLANATION OF FIGURES

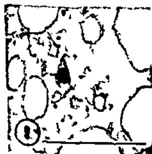
7 A lateral protuberance of an ependymal process containing a variety of dense core vesicles Note that some of the cores appear to consist of two or more granules

8 A dense-core vesicle continuous with a large clear vacuole

9 A dense-core vesicle with lateral tubular expansions of its membrane

10 Two attached dense-core vesicles with complex cores



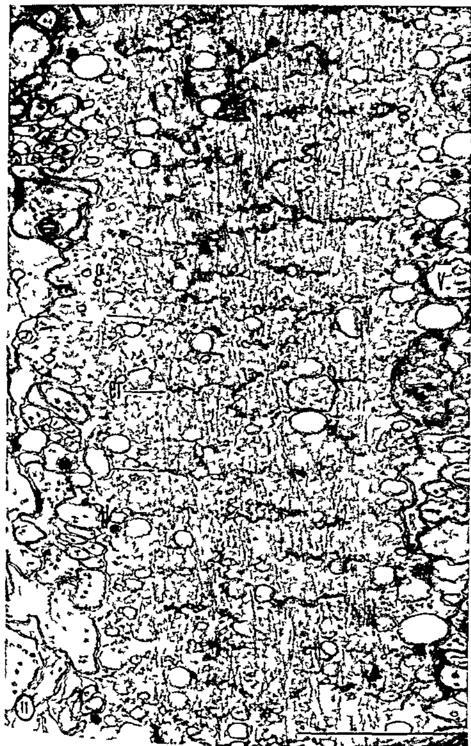




## PLATE 5

### EXPLANATION OF FIGURE

11 Ependymal process displaying a typical central core with bundles of longitudinal filaments and an irregular series of cross striations consisting of membranous plates and vesicular expansions—the ependymal reticulum (er arrows) Large vacuoles (V) display attachments to the ependymal reticulum Glycogen granules (g) and dense core vesicles (d) are irregularly scattered and extend into small lateral protuberances


11

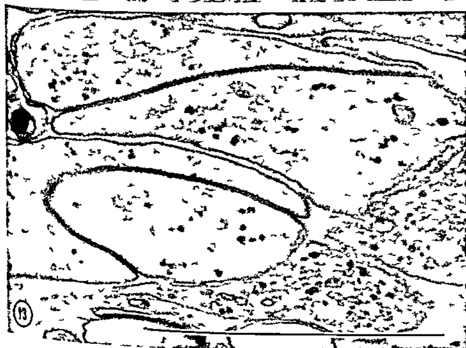
## PLATE 6

### EXPLANATION OF FIGURES

12 Interdigitating finger like processes between a sub pial ependymal end foot (ep) and an adjacent astrocyte process (a) displaying *fasciae occludentes* junctions (o) near the surface of the brain

13 Ependymal tight junctions of the *fascia occludens* variety


ep
a
o
12


13

## PLATE 6

### EXPLANATION OF FIGURES

12 Interdigitating finger like processes between a sub pial ependymal end foot (ep) and an adjacent astrocyte process (a) displaying *fasciae occludentes* junctions (o) near the surface of the brain

13 Ependymal tight junctions of the *fascia occludens* variety

# An Evaluation of Possible Intracellular Nucleic Acid Quenching of Carcinogenic Hydrocarbon Fluorescence [1]

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ABSTRACT An investigation was made to evaluate the role of intranuclear nucleic acids on the reported lack of accumulation of fluorescent polycyclic carcinogenic hydrocarbons (CH) within the nucleus of the live cell Analysis of CH distribution with integrated phase-contrast and u v fluorescent microscopy was performed on whole cells and subcellular nuclear fractions from which nucleic acids had been removed and on ultracentrifuged live cells whose nuclei were stratified into nucleic acid rich and nucleic acid poor regions No intranuclear fluorescence was found in any experimentally treated material or in the live cell controls after their exposure to CH The absence of any evidence for the quenching of CH fluorescence by intranuclear nucleic acids supports the u v fluorescence microscopists contention that little or no CH enters live cell nuclei

The general applicability of the u v fluorescence microscopic technique to the tracing of exogenous polycyclic carcinogenic hydrocarbons (CH) into live cells was first demonstrated by Graffi (39) Subsequent u v fluorescence microscopic studies have shown that the intracellular distribution of CH follows a pattern common to a wide variety of live nucleated vertebrate and invertebrate cell types (Graffi and Mass 38 Gunther 41 Richter and Saini 60 Shires 62 Jones and Harvey et al 63) This pattern shows hydrocarbon accumulation in the general cytoplasmic matrix and a relatively higher accumulation in elements of the Golgi complex and lipid droplets With one possible exception there is no apparent accumulation of CH in the intranuclear area Graffi (39 40) reported the accumulation of CH in the nucleoli of live tumor cells but this has not been confirmed by later studies (Richter and Saini 60)

It is not clear why there is no karyoplasmic localization of CH as assayed by u v fluorescence microscopy both *in vivo* and *in vitro* Purely biochemical studies have shown that bonding of CH with RNA and DNA in aqueous solutions is accompanied by quenching of the CH fluorescence (Boyland and Green 62 64) This would suggest that the apparent absence of CH accumulation by the karyoplasm in living cells is a quenching effect and thus a limiting defect in the u v fluorescence microscopic method However Giovanella et al (64) and Heidelberger (64) have questioned the occurrence of nucleic acid binding with CH both *in vivo* and *in vitro* In this report data are presented which bear on the question of whether the apparent lack of karyoplasmic accumulation of CH is real or whether it is due to the quenching of CH fluorescence by intranuclear RNA and DNA

## MATERIALS AND METHODS

Study of the influence of nucleic acids on the u v fluorescence of intracellular CH involved (1) a depletion of nucleic acids by (a) nuclease digestion of whole fixed cells (b) nuclease digestion and saline extraction of subcellular nuclear fractions and (c) ultracentrifugal displacement and stratification of nucleic acids in live whole cells (2) exposure of this treated material to 3 4 benzo(a)pyrene (BP) (Hoffman LeRoche) (3) examination of intracellular BP patterns and their comparison with those in untreated control material

[1] S pported by a National Institute f Health Pre-Doct l Fellowship

[2] Thi report mpri e p rt of a dissert ti n submitted to the Gr du t F ulty of the Uni ersity of Oklahoma in p rtial fulfillm nt f requi ements f r th degree of Doct r of Philo ophy

which the enzymes had been omitted In the case of DNAase an additional control preparation was made in which the enzyme and magnesium cofactor were jointly omitted In any one preparation control or experimental several coverslips were processed together part to be used for histochemical assay for the extent of nucleic acid removal effected by the phosphodiesterase and part for examination with the carcinogen

*Liver nuclear fractions* Nucleic acids were removed from rat liver nuclei by a modification of the method of Georgiev and Chentsov (62) (1) Nuclear fractions were prepared in 0 25 M sucrose as described above (2) washed three times in McIlvaine s pH 6 buffer (3) digested six hours in 1 mg/ml DNase with 0 005 M MgCl in pH 6 McIlvaine s buffer (4) extracted in 1 M NaCl for 30 minutes in a cold room — the extraction being repeated 9 to 11 times

At steps no 2 no 3 and no 4 samples were withdrawn from the fractions and subdivided for the following procedures (a) observation of nuclei on crystalline beds of BP (b) histochemical tests for nucleic acids (c) measurement of nuclear sizes with a Leitz Ojeckmikrometer and (d) a test of the polymeric state of nucleoprotein in the nuclear fraction by its capacity to form gels in 1 M NaCl (Dounce 55) Nucleoprotein which did not flow down the side of an inverted test tube was designated a strong gel that which showed some flow was arbitrarily considered weak and that which was watery in consistency was designated as not capable of gel formation

### *Methods of BP introduction and observation*

BP was introduced from crystalline beds on glass coverslips or slides formed by evaporation of a saturated ethanol BP stock solution applied by wire loop 20 mm in diameter The various biological materials were exposed to CH as follows (1) Centrifuged or uncentrifuged liver cell suspensions in Eagle s MEM or BME and liver nuclear fractions in sucrose McIlvaine s buffer DNAase solutions or 1 M NaCl were pipetted onto crystalline beds of BP prepared on glass slides (2) Glass cover slips bearing living or experimentally treated L-929 fibroblasts in growth medium or distilled water respectively were inverted and applied to BP crystalline beds on glass slides (3) Crystalline BP beds were prepared on sterile glass coverslips in culture vessels and L-cells suspended in BME or MEM were pipetted into the vessels and cultured for 36–72 hours

Two histochemical procedures were used to assess the presence and location of nucleic acids in experimental and control preparations (a) the Feulgen reaction (Feulgen and Rossenbeck 24 Bauer 32) using Schiff s reagent prepared by the method of Coleman (38) from Hartman and Leddon Basic Fuchsin (b) Gallocyanin Chrom Alum prepared and used according to Einarson (51) with RNAase or DNAase treated controls (Gallocyanin from Matheson Coleman and Bell)

The equipment and methods of the integrated phase-contrast and u v fluorescence microscopic technique were similar to those described in detail by Richter and Saini (60)

## OBSERVATIONS

### *L 929 fibroblasts*

The form of L-cells on glass substrates was predominantly stellate with spindle shaped cells less common The mitochondria were usually rod shaped or granular and occurred at random in the cytoplasm or oriented with the long axis of cell processes (figs 1 3) The Golgi material occured in two forms a condensed juxtanuclear zone (Bensch et al 64) appearing in approximately 33% of the cells (figs 19 25) and a dispersed form composed of small angular structures (Hayward 61) the Golgi granules (figs 1 3) located throughout the cytoplasm L-cells contained a single oval to round nucleus although bi or multinucleate cells and cells with micronuclear fragments were common (figs 1 3) as reported for many other strains of cultured cells (Lewis 47 51 Bucher and Gattiker 53 Ludford 54 Hsu and Moorhead 55 56 Nagata 60 Longwell and Yerganian 65) Nucleoli occurred either as large irregular structures numbering 1 to 3 per nucleus (figs 1 3) or as small compact entities numbering four or more per nucleus (figs

*Preparation of biological material*

*Biological test material consisted of* monolayers of Earle s L 929 mouse fibro blasts (Microbiological Associates) dis sociated but intact liver cells in suspension and subcellular nuclear fractions from rat liver

Routine stocks of strain L 929 fibroblasts were carried in Eagle s MEM or BME with 10% horse serum and a penicillin strepto mycin mixture (Microbiological Associates or Flow Labs) Experimental studies were carried out on coverslip cultures grown in disposable petri dishes (Falcon Plastics) Cells were seeded at approximately 200 000 per ml and allowed to grow 2 to 4 days

Whole liver cells were obtained from King Holtzman rats sacrificed by cervical dislocation Small fragments of liver were quickly excised and immersed in Eagle s MEM or BME in a physiologically clean petri dish Fragments were minced with scissor type strokes of knives fitted with no 15 Bard Parker knives until a turbid suspension of dissociated cells was ob tained

*Subcellular nuclear fractions* of King Holtzman rat livers were isolated in 0 25 M sucrose by a procedure similar to those described by Hogeboom et al (62) and Allfrey et al (57) Homogenization was done *in 0 25 M sucrose with 0 0018 M* $CaCl_2$ made up in triple distilled water (Cutter) Ten milliliters of medium was used per gram of tissue The homogenate was filtered through 4 to 6 layers of sterile *surgical gauze and the filtrate centrifuged* at 150 × G for ten minutes in an Inter national Clinical Centrifuge Model CL to sediment the unbroken cells The super natant was then spun at approximately 380 × G for ten minutes the sediment represented the nuclear fraction Resus pension and resedimentation of the nu clear fractions were done several times to remove as much non nuclear material as possible All steps were carried out either *in a cold room (2°) or in an ice bath*

*Removal or displacement of nucleic acids*

*Ultracentrifugation* Ultracentrifugation of liver tissue was accomplished with a Beam s air turbine centrifuge (J W Bear '30) *Constant* pressure air supply provided by a Quincy air-compres (Model 325) rated at 22 cubic feet minute and driven by a five hp motor

Clean aluminum rotors were load with 0 5–0 8 cm³ of Eagle s BME or MI and 3 to 5 liver fragments freshly exci and reduced to dimensions of appro mately 0 3–0 5 cm by 1 to 2 cm Cent fugal forces of 300 000 or 400 000 × were maintained on the tissue for one o and a half or two hours Calculation centrifugal forces was based on pressu rotational speed curves prepared for t rotors by Fritz Linke of the Physical Lab atory University of Virginia

*In vitro* preparations of centrifug liver were prepared by mincing the fr ments in the same manner as was d scribed above for the uncentrifuged liv Portions of the centrifuged tissue we fixed in formalin and sectioned in paraff for histochemical tests

*L 929 fibroblasts* Removal of nucle acids from L-cells was carried out aft fixation in Zenker s fluid (one hour) a *conventional removal of* mercuric chlori with Lugol s solution and sodium thiosu fate (Cowdry 48)

Deoxyribonuclease (DNAase) (Wort ington, 1X crystallized) was used in M *Ilvaines buffer (0 1 M citrate and 0 2* phosphate) at pH 6 0 (Kunitz 50 Korso 51 Daoust and Clermont 55) with 0 0 M $MgCl_2$ (Kunitz 50) The enzyme co centrations employed were 0 2 0 6 1 *and 2 0 mg/ml One mg%* chloramphe icol (Parke Davis) was added to incub tion media when the enzyme concentr tion exceeded 1 mg/ml Incubation laste 3 to 6 hours at 37° The cells were fixe and digested directly on their origin coverslip substrates

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Control preparations for both DNAa and RNase were incubated in media fro

in living preparations (Beams and King 38 Claude 43 Lagerstedt 49 Brenner 53 Bernhard et al 54 Kopac 55) Gallocyanin-Chrom Alum staining after DNAase hydrolysis and Feulgen staining demonstrated that RNA and DNA occurred in the chromatin (figs 33 34) In agreement with Brenner (53) some nuclear membrane associated chromatin did not sediment even under forces of 200 000 × G for two hours (figs 33 34) Interchromatin regions especially in the centripetal portions of the nucleus appeared to be free of histochemically demonstrable nucleic acid

The distribution of BP in ultracentrifuged cells in comparison with uncentrifuged controls suggested that the displacement of cell organelles had no effect on their affinity for BP Lipid droplets and the small granule component showed high concentrations of the CH and mitochondria relatively little uptake of BP (figs 36 38) Displacement of nuclear chromatin also had no effect in altering the lack of affinity of all intranuclear structures for CH (figs 36 38)

*Liver cell nuclei*

Pellets of liver nuclei fractionated in 0.25 M sucrose-0 0018 M $CaCl_2$ were always contaminated with cytoplasmic material. The nuclei resembled those prepared by Roodyn (63) in being circumscribed by a dark nuclear rim and containing one to several dark compact nucleoli in nearly hyaline non nucleolar karyoplasm (figs 39 41) Resuspension of the nuclei in pH 6 0 McIlvaine's buffer and incubation for six hours at 37 produced (a) coalescence of the non nucleolar karyoplasm (b) the appearance of blebs on the nuclear rim and (c) the agglutination of cytoplasmic debris on the nuclear rim (fig 43) These characteristics were also found when $MgCl_2$ was added to the incubation buffer (figs 45 47) Weak nucleoprotein gels were formed by nuclear fractions incubated in buffer whereas strong gels having little or no flow were formed by the fresh sucrose preparations (table 1)

Resuspension and incubation of sucrose isolated nuclei in DNAase caused the loss of all Feulgen stainable material and the appearance of clear areas in the non nucleolar karyoplasm as found by Anderson (53b) (figs 49 51) Sequential treatment with DNAase and 1M NaCl produced poor yields of shrunken distorted nuclei In these nuclei the nuclear rim was connected to the residual nucleolus by a few strands of dark material (figs 53 56 58) The average nuclear size was less than one third that of the untreated nuclei in sucrose (table 1) Staining with the Feulgen procedure or Gallocyanin Chrom Alum was not observed in any of the exhaustively extracted nuclei (table 1)

BP accumulations in liver nuclei prepared in 0 25 M sucrose were limited to the nuclear rim (figs 40 42) confirming the findings of Norden (57) No demonstrable BP occurred within the confines of the nuclear rim in Feulgen negative nuclei incubated in DNAase (figs 50 52) or in buffer with or without magnesium (figs 44 46 48) No apparent CH concentration occurred in nuclear blebs except in their rims (figs 44 46 50) Fluorescence occurring over the dark central non fluorescent intranuclear region appeared referable to localizations of BP in cytoplas

TABLE 1

*Characteristics of treated liver nuclei*[1]

| Treatm nt | Feulgen | Gel | Nuclear dimensions[2] |
|---|---|---|---|
| 1. In 0 25 M sucrose and 0 0018 M CaCl | yes | strong | 11 0(±2 55) × 8 7(±2 26) |
| 2. In McIlvaine's B six hours without Mg | yes | weak | 5 7(±1.36) × 5.2(±0 85) |
| 3. In McIlvaine's B six hours with Mg | yes | weak | 5 3(±1 04) × 5 0(±0 92) |
| 4. In DNAase solution | no | none | 5 6(±1 26) × 5 5(±0 64) |
| 5. In DNAase followed by 1 M NaCl | no | none | 3 8(±0 11) × 3 6(±0 05) |

[1] Observ tions were carried out on more than 600 nu lei per procedure except those in the DNAa e-1 M NaCl prep ration where only 53 nuclei in fi prep rations were found

[2] The n le dimensions and standard de i ti ns are all in mic

5 7) Vacuolation (Lewis 43) occurred in the large nucleoli (figs 5 9)

The distribution of BP in living L cells was similar in cells exposed to the CH crystals for a few moments or cultured on BP several days Accumulations of CH were relatively greatest in components of the Golgi apparatus (figs 2 4) Mitochondria displayed lower apparent BP concentrations than occurred throughout the general cytoplasmic matrix (fig 2) All nuclear structures including nuclear membrane nucleoli chromatin and micronuclei showed no demonstrable accumulation of BP

Fixation of L cells with Zenker s fluid produced a coarsely precipitated cytoplasm in which no mitochondria were observed (Strangeways and Canti 28) Lipid droplets and vacuoles appeared much more commonly in the fixed cells than in the living (figs 5 7) Golgi material preservation was apparently variable (cf figs 11 13 vs figs 5 7) The chromatin material was finely precipitated and more discrete than in living cells both chromatin and nucleoli tended to be relatively more refractile than in life (figs 5 7) A dense precipitation of cytoplasmic and nucleoplasmic material in the nuclear membrane region was characteristic of Zenker s fluid fixation (figs 5 7)

No alteration in the phase contrast microscopic morphology of Zenker s fluid fixed cells was incurred when mercuric chloride was removed with Lugol s solution and thiosulfate (fig 9) No apparent changes in Zenker s fluid fixed cytology occurred when cells were (a) incubated in McIlvaine s buffer six hours (fig 11) (b) digested with DNAase with or without the magnesium cofactor (figs 13 15 17 19) so that Feulgen staining was totally abolished (c) digested with RNAase so that Gallocyanin Chrom Alum staining of the cytoplasm was absent and nuclear staining reduced (figs 21 23) or (d) sequentially incubated with both phosphodiesterases until Feulgen and Gallocyanin Chrom Alum staining were absent or greatly reduced (figs 25 27) No depletion in the amount of precipitated chromatin material was noticed although a substantial diminution of histochemically demonstrable nucleic acid was obtained (figs 13 15 17 1[illegible] 21 23)

The pattern of intracellular BP appear[illegible] similar in the enzyme treated and u[illegible] treated Zenker s fluid fixed cells Gol[illegible] material and lipid droplets demonstrat[illegible] the relatively highest BP accumulatio[illegible] while lower CH concentrations were loc[illegible] ized in the cytoplasmic vacuoles in cel[illegible] digested with DNAase (with or witho[illegible] magnesium — figs 14 16 18 20) wi[illegible] RNAase (figs 22 24) with RNAase an[illegible] DNAase (figs 26 28) or in cells simpl[illegible] fixed in Zenker s fluid with or without re[illegible] moval of mercuric chloride (figs 6 [illegible] 10) No demonstrable BP was localize[illegible] in any nuclear structure in cells deplete[illegible] of DNA (figs 14 16 18 20) RNA (fig[illegible] 22 24) DNA and RNA (figs 26 28) [illegible] in cells in which the nucleic acid complement was intact (figs 6 8 10 12)

*Hepatic paraenchymal cells*

In living rat hepatic parenchymal cell[illegible] the nuclei contained discrete nuclear mem[illegible] branes one to several irregular shaped dar[illegible] nucleoli and irregular strands of chromati[illegible] (Zollinger 48 Bucciolini and Marsili 60 Colosi and Marsili 60) (figs 29 31) In the cytoplasm mitochondria lipid drop[illegible] lets and a fine granular component of un[illegible] certain nature were distinguishable (figs 29 31) Intracellular BP was not local[illegible] ized in any nuclear structure or in the[illegible] mitochondria accumulations of the CH occurred in lipid droplets and the smal[illegible] granule component in relatively greater concentrations than those in the general cytoplasmic matrix (figs 30 32)

Living ultracentrifuged liver cells ob[illegible] served in slide preparations failed to con[illegible] sistently show discrete cytoplasmic strata in contrast to three or four strata found on fixed sections of the same material and as reported by Beams and King (34) Claude (43) Lagerstedt (49) Bernhard et al (54) and Kopac (55) The indis[illegible] tinctness of the cytoplasmic strata was probably due to relocation of components during the preparation after centrifugation (Mateyko and Kopac, 63)

Most nuclear chromatin in ultracentri[illegible] fuged liver cells was found sedimented against the centrifugal nuclear pole (figs 33 34) as previously described for fixed

or lung preparations (Beams and King 34 Claude 43 Lagerstedt 49 Brenner 53 Bernhard et al 54 Kopac 55) Gallocyanin-Chrom Alum staining after DNAase hydrolysis and Feulgen staining demonstrated that RNA and DNA occurred in the chromatin (figs 33 34) In agreement with Brenner (53) some nuclear membrane associated chromatin did not sediment, even under forces of 200 000 × G for two hours (figs 33 34) Interchromatin regions especially in the centripetal portions of the nucleus appeared to be free of histochemically demonstrable nucleic acid.

The distribution of BP in ultracentrifuged cells in comparison with uncentrifuged controls suggested that the displacement of cell organelles had no effect on their affinity for BP Lipid droplets and the small granule component showed high concentrations of the CH and mitochondria relatively little uptake of BP (figs 36 38) Displacement of nuclear chromatin also had no effect in altering the lack of affinity of all intranuclear structures for CH (figs 36 38)

*Liver cell nuclei*

Pellets of liver nuclei fractionated in 0.25 M sucrose-0 0018 M $CaCl_2$ were always contaminated with cytoplasmic material. The nuclei resembled those prepared by Roodyn (63) in being circumscribed by a dark nuclear rim and containing one to several dark compact nucleoli in nearly hyaline non nucleolar karyoplasm (figs 39 41) Resuspension of the nuclei in pH 6 0 McIlvaine's buffer and incubation for six hours at 37 produced (a) coalescence of the non nucleolar karyoplasm (b) the appearance of blebs on the nuclear rim and (c) the agglutination of cytoplasmic debris on the nuclear rim (fig 43) These characteristics were also found when $MgCl_2$ was added to the incubation buffer (figs 45 47) Weak nucleoprotein gels were formed by nuclear fractions incubated in buffer whereas strong gels having little or no flow were formed by the fresh sucrose preparations (table 1)

Resuspension and incubation of sucrose isolated nuclei in DNAase caused the loss of all Feulgen-stainable material and the appearance of clear areas in the non nucleolar karyoplasm as found by Anderson (53b) (figs 49 51) Sequential treatment with DNAase and 1M NaCl produced poor yields of shrunken distorted nuclei In these nuclei the nuclear rim was connected to the residual nucleolus by a few strands of dark material (figs 53 56 58) The average nuclear size was less than one third that of the untreated nuclei in sucrose (table 1) Staining with the Feulgen procedure or Gallocyanin-Chrom Alum was not observed in any of the exhaustively extracted nuclei (table 1)

BP accumulations in liver nuclei prepared in 0 25 M sucrose were limited to the nuclear rim (figs 40 42) confirming the findings of Nordén (57) No demonstrable BP occurred within the confines of the nuclear rim in Feulgen negative nuclei incubated in DNAase (figs 50 52) or in buffer with or without magnesium (figs 44 46 48) No apparent CH concentration occurred in nuclear blebs except in their rims (figs 44 46 50) Fluorescence occurring over the dark central non fluorescent intranuclear region appeared referable to localizations of BP in cytoplas-

TABLE 1
*Characteristics of treated liver nuclei*[1]

| Treatment | Feulgen | Gel | Nuclear dimensions[2] |
|---|---|---|---|
| 1. In 0.25 M sucrose and 0 0018 M CaCl | yes | strong | 11 0(±2 5.) × 8 7(±2 26) |
| 2. In McIlvaine's B six hours without Mg | yes | weak | 5 7(±1.36) × 5.2(±0.85) |
| 3. In McIlvaine's B six hours with Mg | yes | weak | 5.3(±1 04) × 5 0(±0.92) |
| 4. In DNAase solution | no | none | 5 6(±1 26) × 5 5(±0 64) |
| 5. In DNAase followed by 1 M NaCl | no | none | 3 8(±0 11) × 3 6(±0 05) |

[1] Observation were carried out on more than 600 nuclei per procedure except those in the DNAase-1 M NaCl preparations where only 53 nuclei in five preparations were found
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The distribution of BP in living L cells was similar in cells exposed to the CH crystals for a few moments or cultured on BP several days Accumulations of CH were relatively greatest in components of the Golgi apparatus (figs 2 4) Mitochondria displayed lower apparent BP concentrations than occurred throughout the general cytoplasmic matrix (fig 2) All nuclear structures including nuclear membrane nucleoli chromatin and micronuclei showed no demonstrable accumulation of BP

Fixation of L-cells with Zenker s fluid produced a coarsely precipitated cytoplasm in which no mitochondria were observed (Strangeways and Canti 28) Lipid droplets and vacuoles appeared much more commonly in the fixed cells than in the living (figs 5 7) Golgi material preservation was apparently variable (cf figs 11 13 vs figs 5 7) The chromatin material was finely precipitated and more discrete than in living cells both chromatin and nucleoli tended to be relatively more refractile than in life (figs 5 7) A dense precipitation of cytoplasmic and nucleoplasmic material in the nuclear membrane region was characteristic of Zenker s fluid fixation (figs 5 7)

No alteration in the phase contrast microscopic morphology of Zenker s fluid fixed cells was incurred when mercuric chloride was removed with Lugol s solution and thiosulfate (fig 9) No apparent changes in Zenker s fluid fixed cytology occurred when cells were (a) incubated in McIlvaine s buffer six hours (fig 11) (b) digested with DNAase with or without the magnesium cofactor (figs 13 15 17 19) so that Feulgen staining was totally abolished (c) digested with RNAase so that Gallocyanin Chrom Alum staining of the cytoplasm was absent and nuclear staining reduced (figs 21 23) or (d) sequentially incubated with both phosphodiesterases until Feulgen and Gallocyanin Chrom Alum staining were absent or greatly reduced (figs 25 27) No depletion in the amount of precipitated chromatin material was noticed although a substantial diminution of histochemically demonstrable nucleic acid was obtained (figs 13 15 17 F 21 23)

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Living ultracentrifuged liver cells observed in slide preparations failed to consistently show discrete cytoplasmic strata in contrast to three or four strata found on fixed sections of the same material and as reported by Beams and King (34) Claude (43) Lagerstedt (49) Bernhard et al (54) and Kopac (55) The indistinctness of the cytoplasmic strata was probably due to relocation of components during the preparation after centrifugation (Mateyko and Kopac 63)

Most nuclear chromatin in ultracentrifuged liver cells was found sedimented against the centrifugal nuclear pole (figs 33 34) as previously described for fixed

### *The retention and assay of nucleic acids*

Existing literature suggests complete removal of nucleic acids from cells is not achieved with either nuclease or salt extraction DNA reportedly remains in liver sections fixed in any of several fluids including Zenkers after DNAase hydrolysis (Ficq and Errera 58) Isolated unfixed thymic nucleic acids have variously been reported to retain about one fourth of their total DNA content after DNAase treatment (Allfrey et al 57 Allfrey and Mirsky 58 Naora et al 61) Some RNA remains in unfixed nuclei after exhaustive enzymatic and salt extractions (Georgiev 58 Zbarski and Georgiev 59 Smetana et al 63 Steele and Busch 63)

The Feulgen reaction as a method for assessing DNA reportedly has a high threshold of sensitivity (Widstrom '28 Caspersson '32 Ris and Mirsky 49 Dische 55) The binding of Gallocyanin Chrom Alum with either DNA or RNA occurs stoichiometrically but apparently no precise data on the nucleic acid concentration range demonstrable with the Einarson technique has been presented (Sandritter et al 63)

The limitations in histochemical sensitivity as well as the incomplete extractability of nucleic acids mean that nuclei totally free of nucleic acids probably have not been obtained in the present study Presumably if CH fluorescence is quenched by intranuclear nucleic acids a reduction in the nucleic acid concentrations to or below the sensitivity thresholds for the Feulgen and Gallocyanin Chrom Alum tests would result in the appearance of a faint intranuclear fluorescence

## CONCLUSIONS

Cells and their nuclei submitted to three different experimental procedures for altering nucleic acid content at specific cell sites consistently showed no apparent BP accumulations in intranuclear material This invariable pattern of BP compartmentalization is similar to that observed in living cells relative to the nucleus The evident lack of dependence of the BP pattern on nucleic acid content or distribution suggests that the nucleic acid associated quenching of BP fluorescence found by Boyland and Green (62) using biochemical techniques may not occur in biologic structures such as the nucleus

Berg (51 52) hypothesized the lack of intranuclear accumulation of BP was related to the absence of lipid in the nucleus There are however numerous biochemical and histochemical reports of lipid in the nucleus in chromosomes — both mitotic and interphase (Claude and Potter 43 Ris and Mirsky 49 Cohen 49 Brock et al 52 Idelman 57 58a b Chayen et al 57 59 LaCour et al 58) in nucleoli (Brock et al 52 Zagury 57 Barer and Dick 57 Georgiev 58 Zbarski et al 62 Georgiev and Chentsov 62) in the nuclear membrane (Schmitt '38) and in whole nuclei (Stoneburg '39 Hack 48 Engbring and Laskowski 53 Wang et al 53 Dounce 55 Chauveau et al 56 Levin and Thomas 61) Experimental lipid extraction of cells generally supports Berg's position that lipid determines the distribution of CH in the cytoplasm (Shires 65b) but at present there is no data on which to base an explanation for the lack of affinity for BP by cell sites which apparently contain lipid

The lack of intranuclear fluorescence in live cells exposed to BP may indicate low levels of BP but not its total absence from the nucleus While concentrations near those in serum (60–70 µg/ml in calf serum — Morimura et al 64) are perceptible with u v fluorescence microscopy (Richter and Saini 60) lower concentrations perhaps would be indiscernible in the nucleus Reactions between CH and cell constituents would seem more likely in sites where apparently high CH concentrations occur e g the cytoplasm than in regions where at most very low concentrations are suspected Of the various proposed mechanisms of chemical carcinogenesis u v fluorescence microscopic data is not consistent with direct interaction between CH and chromosomal material (e g Haddow 58) but does support theories which allow primary CH reactions to occur at the edge of the nucleus or in the cytoplasm (e g Pitot and Heidelberger 63)

## ACKNOWLEDGMENTS

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mic debris on the nuclear rim Liver nu clei sequentially treated with DNAase and 1 M NaCl displayed high accumulations of CH *in the nuclear rim* but no demon strable BP in the nuclear interior (figs 54 55, 57 59) The pattern of BP distribu tion in all experimentally treated isolated liver nuclei was similar to the pattern of BP relative to the nucleus in live hepatic parenchymal cells

## DISCUSSION

No demonstrable intranuclear BP was found in live fibroblasts and hepatic cells or in nuclear fractions from which nucleic acids had been removed or displaced These results were obtained despite the cytologic alterations resulting from the side effects of the procedures for nucleic acid removal

Fixation of fibroblasts was necessary for the long enzymatic hydrolysis and was achieved using Zenker s fluid fixation which was compatible with nuclease activ ity (cf Brachet 53, Pearse 60) and caused little alteration in the live cell pat tern of intracellular BP Its selection was made after preliminary studies previously reported in part (Shires, 65a) The effect of Zenker s fluid fixation in agglutinating material to the nuclear membrane and in precipitating the karyoplasm also seen by Strangeways and Canti ('28) probably led to the common incidence of CH fluorescence imposed on the nuclear profile by highly BP rich cytoplasmic structures above or below the nuclear rim No noticeable change in the appearance of Zenker s fluid fixed protoplasm was found after in cubation with RNAase DNAase or the buffer without enzyme

The DNAase incubation medium used on rat liver nuclear fractions contained con stituents in addition to the enzyme which altered the appearance of nuclei isolated in sucrose solutions Suspension and in cubation of nuclei in McIlvaine s buffer with or without Mg led to changes de scribed by others for Mg phosphate or citrate ions at concentrations similar to those in the incubation medium (1) con densation of the nucleoplasm (Bernstein and Mazia 53, Philpott and Stanier, 56 57 Schneider 55 Roodyn, 63) (2) promotion of the appearance of blebs from the nuclear rim (Anderson 53a) (3) de crease in nuclear volume (Anderson and Wilbur 52) The incubation mixture also contained cytoplasmic contaminants of the nuclear fraction which probably contri buted some DNAase activity (Dounce et al 57)

Exhaustive nucleic acid extraction by the sequential treatments of isolated nu clei with phosphodiesterase and 1 M NaCl entails removal of chromosomal and peri chromosomal globulins DNA, histones and the tryptophane rich proteins (Mirsky and Pollister, 46 Mirsky and Ris 50 51 Georgiev, 58 Zbarski and Georgiev 59 Zbarski et al 62 Georgiev and Chentsov 62 Steele and Busch 63 Busch et al 63) The removal of these substances probably leaves some unextractable ribonu cleoprotein uncharacterized lipid and an acidic protein (Mirsky and Pollister, 46 Wang et al 53 Georgiev 58 Zbarski and Georgiev 59 Zbarski and Ermolaeva 60 Zbarski et al 62 Georgiev and Chentsov 62 Steele and Busch, 63 Busch et al 63) in which BP could ac cumulate The phase contrast photomicro- graphs of the present study and the elec tron micrographs of Zbarski et al (62) and Georgiev and Chentsov (62) suggest that most of this residual material is lo- cated in the nuclear rim

In contrast to the nucleic acid removal procedures ultracentrifugal dislocation of chromatin represents an alteration not harmful even to highly differentiated cell types (Guyer and Claus 36a b Mac Dougald et al 37 Dornfeld 36 37 Mateyko and Kopac 63) The results of this mild procedure on the intracellular distribution of BP confirm those of Wad dington and Goodhart (49) on oocytes and gastrular cells of *Triturus alpestris*, using BP solubilized with caffeine The similarity of the nuclear BP patterns in centrifuged rat liver cells with those in nucleic acid depleted L-cells and nuclear fractions suggests that the technical altera tions involved in the nuclease or extrac tion procedures did not influence the dis tribution of BP

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All the figures are ordinary light (LPM) dark-contrast medium phase (PCM) or u v fluorescence (UVPM) photomicrographs taken with a 35 mm Leica camera with a Micro-Ibso attachment and a 1/3dx conical ex tension tube All figures are 1 700 X *magnification*

## PLATE 1

### EXPLANATION OF FIGURES

1 Two live L-cells approximately five minutes after contact with BP crystals displaying Golgi granules (G) and mitochondria (M) in the cytoplasm and nucleoli (nc) chromatin (Ch) and nuclear mem brane (Nm) as parts of the nucleus A *micronuclear fragment (Mn)* is seen in the upper cell PCPM

2 Same cells as in figure 1 in which BP concentrations are highest in *Golgi granules (G) but absent in mitochondria (M)* and nuclei (N) UVPM

3 Two L-cells grown on BP crystals for about 60 hours showing Golgi granules (G) a micronucleus (Mn) and nuclear detail including nucleoli (nc) PCPM

4 The same cells as in figure 3 showing no BP uptake by any nuclear structure (N) or the micronucleus (Mn) but relatively high uptake by the Golgi granules (G) UVPM

5 Portions of two L-cells fixed in Zenker s fluid before exposure to BP Nucleoli (nc) are refractile A protoplasmic precipitate has ag gregated in the region of the nuclear membrane (nm) Vacuoles occur in the cytoplasm (V) PCPM

6 The same cells as in figure 5 displaying no discrete BP accumula tions in the whole nuclear region (N) or in cytoplasmic vacuoles (V) UVPM

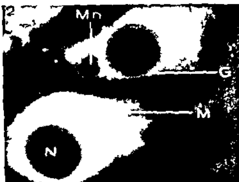
7 L-cells from a culture grown on BP for 48 hours fixed in Zenker's fluid showing fine chromatin in the nucleus (Ch) and a heavy deposit of material in the vicinity of the nuclear membrane (nm) PCPM

8 The same cells as in figure 7 in which no BP accumulation is apparent within the nucleus (N) UVPM

9 A large spindle shaped cell grown on BP crystals *in vitro* fixed in Zenker's fluid and treated with Lugol s solution and thiosulfate Highly refractile nucleoli (nc) fine chromatin precipitate (Ch) and cytoplasmic *vacuoles (V) are indicated PCPM*

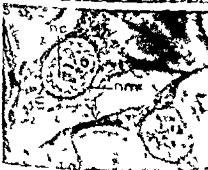
10 The same cell as in figure 9 in which no intranuclear accumulation of BP is demonstrable (N) Cytoplasmic vacuoles (V) also appear free of the CH UVPM


m
nc

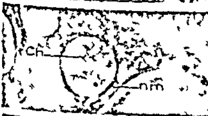

2
Mn
G
M
N


Mn
G
nc

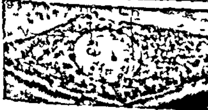

4
Mn
N
G


5
nc
nm


N
V


Ch
nm


8
N


9
V
nc
Ch


10
V
N

All the figures are ordinary light (LPM) dark-contrast medium phase (PCM) or u v fluorescence (UVPM) photomicrographs taken with a 35 mm Leica camera with a Micro-Ibso attachment and a 1/3dx conical extension tube All figures are 1 700 × magnification

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8 The same cells as in figure 7 in which no BP accumulation is apparent within the nucleus (N) UVPM

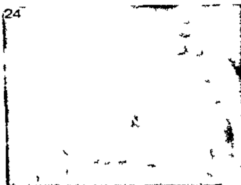
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10 The same cell as in figure 9 in which no intranuclear accumulation of BP is demonstrable (N) Cytoplasmic vacuoles (V) also appear free of the CH UVPM


21
nc


22


23
nc
Ch


24


25
nc
Gz
Ch


26
N
Gz


Ch
Ch


N

## PLATE 3

### EXPLANATION OF FIGURES

21 A flattened L fibroblast exposed to BP after incubation in RNAase *The residue of several nucleoli (nc) are seen in the nucleus PCPM*

22 The same cell as in figure 21 No BP uptake is associated with any specific intranuclear structure (N) UVPM

23 Several L-cells digested in RNAase after growing on crystalline BP for approximately 48 hours Nucleolar (nc) and chromatin (Ch) residues are visible within the nucleus PCPM

24 The same cells as in figure 23 No BP accumulations appear localized in any intranuclear structure fluorescence over the nuclear region appears referable to cytoplasmic structures above or below the nucleus UVPM

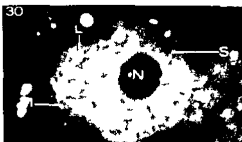
25 Two L-cells exposed to a crystalline bed of BP after successive digestions in DNAase and RNAase A Golgi zone (Gz) is seen in the cytoplasm of one cell and the remnants of nucleoli (nc) and chromatin (Ch) are seen within both nuclei PCPM

26 The same cells as in figure 25 showing relatively high BP concentrations in the Golgi zone (Gz) but no BP localization in any discrete intranuclear structure (N) UVPM

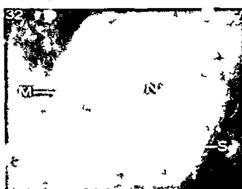
27 Several L-cells grown approximately 48 hours on BP prior to consecutive digestions in DNAase and RNAase No Feulgen stainable material was present in the chromatin residue (Ch) and only a faint diffuse staining with Gallocyanin Chrom Alum was present in the nucleus ICPM

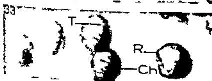
28 The same cells as in figure 27 showing no apparent BP uptake by any intranuclear structure (N) UVPM

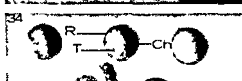

L
Sg
Ch
M

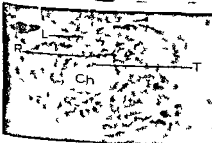

30
L
S
N


nc
Sg


32
M
N
S

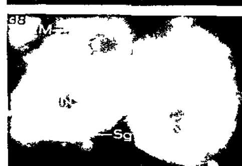

33
T
R
Ch


34
R
T
Ch


L
R
T
Ch


36
L
N


M
Sg


38
M
N
Sg

## PLATE 4

### EXPLANATION OF FIGURES

29 A dissociated heptic parenchymal cell showing lipid droplets (L) swollen mitochondria (M) the small granular component (Sg) and nuclear chromatin (Ch) PCPM

30 Same cell as in figure 29 with demonstrable BP accumulated in lipid droplets (L) and the small granule component (Sg) but no fluorescence *within the nucleus (N) or mitochondria (M)* UVPM

31 A dissociated binucleate rat liver cell *in vitro* with mitochondria (M) and the small granule component (Sg) discernible in the cytoplasm and nucleoli (nc) in the nucleus PCPM

32 Same cell as in figure 31 demonstrating BP uptake in the small granule component (Sg) and relatively no BP localization in nuclei (N) or mitochondria (M) UVPM

33 Paraffin section of ultracentrifuged liver stained with the Feulgen reaction Most chromatin (Ch) is in the centrifuged nuclear region connected by chromatin threads (T) with unsedimented DNA (R) LPM

34 Paraffin section of ultracentrifuged rat liver stained with Gallocyanin Chrom Alum after treatment with DNAase RNA is demonstrated in the sedimented chromatin (Ch) connecting threads (T) and the unsedimented rim (R) LPM

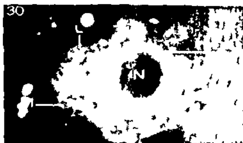
35 A dissociated ultracentrifuged liver cell *in vitro* showing the centrifugal *accumulation of chromatin (Ch) the connecting threads* (T) and the centripetal undisplaced chromatin (R) PCPM

36 Same cell as in figure 35 displaying high BP concentrations in lipid droplets (L) but little in the whole nuclear region (N) UVPM

37 A pair of binuclear ultracentrifuged liver cells with mitochondria (M) the small granule component (Sg) and centrifugally displaced chromatin (Ch) PCPM

38 Same cells as in figure 37 showing no apparent BP accumulation in the nucleus (N) or mitochondria (M) but relatively high BP concentrations in the small granule component (Sg) UVPM

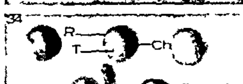

L
Sg
Ch


30
L
N
I


T
nc
Sg


32
M
N


33
T
R
Ch


34
R
T
Ch


L
R
T
Ch


36
L
N


Sg


38
M
Sg

PLATE 5

EXPLANATION OF FIGURES

39 Several rat liver nuclei in 0 25 M sucrose 0 0014 M $CaCl_2$ The nuclear rims (R) and nucleoli (nc) are discernible PCPM

40 The same nuclei as in figure 39 showing localization of BP only in the nuclear rim (R) BP uptake is also seen in the non nuclear debris UVPM

41 Rat liver nuclei viewed in 0 25 M sucrose on crystalline BP Some condensation of the karyoplasmic material (Ky) is evident UVPM

42 The same nuclei as in figure 41 showing the apparent absence of BP in all nuclear *structures (N) except the nuclear rim (R) PCPM*

43 A single nucleus with nucleoli (nc) and a number of nuclear blebs (B) from a preparation incubated at 37 for six hours in McIlvaine s buffer PCPM

44 The same nucleus as in figure 43 showing no BP localization by nuclei (N) or blebs (B) except in their rims (R) UVPM

45 A rat liver nucleus and its blebs (B) from a preparation incubated in pH 6 McIlvaine s buffer with magnesium for six hours prior to exposure to BP PCPM

46 The same nucleus as in figure 45 showing no fluorescence in the nucleus (NO) or its blebs (B) except in their rims (R) UVPM

47 A single rat liver nucleus from a preparation similar to that of figure 45

48 The same nucleus as in figure 47 showing no apparent intranuclear BP (N) The *nuclear rim and adherent cytoplasmic debris accumulate BP (R)*

49 A Feulgen negative nucleus after digestion with DNAase and exposure to BP Nucleoli (nc) blebs and clear spaces in the non nucleolar karyoplasm are demonstrated PCPM

50 The same nucleus as in figure 49 showing no BP accumulation within the nucleus (N) and BP uptake by the rim UVPM

51 Two Feulgen negative nuclei after DNAase treatments with discernible nucleoli (nc) PCPM

52 The same cells as in figure 51 showing no BP uptake by material within the confines of the rim (R) UVPM

53 A rat liver nucleus after both DNAase and 1 M NaCl extraction The rim (R) has much adherent debris PCPM

54 The same nucleus as in figure 53 showing BP uptake only in the rim (R) UVPM

55 A nucleus from a similar preparation to that in figure 53 Apparent BP localization is limited to the nuclear rim and adherent debris (C) UVPM

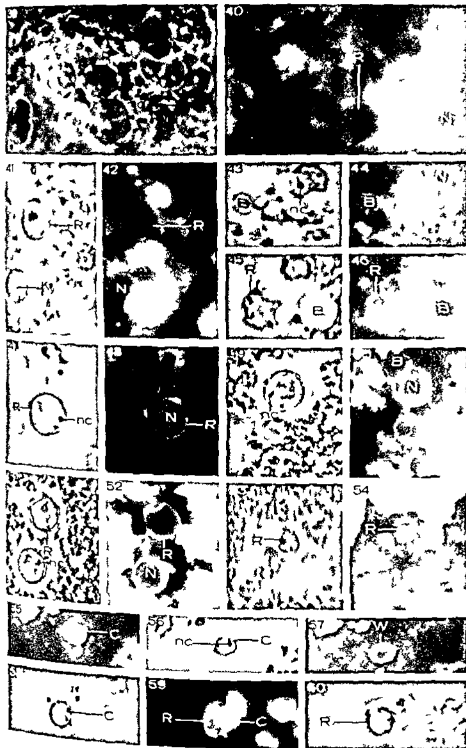
56 The same nucleus as in figure 55 showing the presence of a residual nucleolus (nc) PCPM

57 The *same cell as in* figures 55 and 56 showing BP uptake in a wrinkle of the nuclear rim (W) UVPM

58 A liver nucleus after DNAase and exhaustive 1 M NaCl extraction A thick rim with adherent debris (C) surrounds the nucleus PCPM

59 The same nucleus as in figure 58 displaying BP accumulation only in the rim (R) and cytoplasmic debris (C) UVPM

60 The same nucleus as in *figure 58 as a lower focal plane* PCPM


40
41
R
42
R
N
43
B
nc
44
B
R
B
46
R
B
R
nc
N
R
B
N
nc
R
nc
52
R
N
R
54
R
55
C
56
nc
C
57
W
C
59
R
C
60
R

# Placentation of the Sea Otter [1]

AKHOURI A SINHA AND H W MOSSMAN
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ABSTRACT Development of the sea otter placenta is described from implantation to term. It is a typical carnivore placenta labyrinthine endotheliochorial and zonary (sometimes incomplete mesometrially) The annulus has 4 to 8 lobes and is widest antimesometrially Maternal capillaries of the labyrinth have very thick almost columnar endothelium and thick diastase resistant, heavily PAS positive basement membranes They are surrounded by syntrophoblast and a few cytotrophoblast cells Instances of intratrophoblastic fetal capillaries increase with age thus maternal and fetal blood are in greater proximity in late gestation The hematome arises during the limb-bud period at the center of the antimesometrial portion of the developing chorio-allantoic placenta as small isolated pouches of vascular chorio-allantoic membrane filled with maternal blood Each pouch is lined by columnar cytotrophoblast which phagocytoses maternal erythrocytes and no doubt also absorbs histiotrophic products of the endometrial glands at its base These early pouches grow and coalesce to form larger ones The definitive hematome consists of one large often bilobed sac (diam. 5 to 7 cm) surrounded at the base of its stalk by several smaller sacs Elaborate villous folds subdivide the sacs and stalk The whole hematomic mass invaginates the allantoic vesicle the endoderm of which thus becomes its outer covering The hematome remains very large to term The uterine glands of the placental base are highly active throughout gestation

The sea otter *Enhydra lutris* (Linnaeus) inhabits the coastal waters of the North Pacific Ocean and is the only living marine mustelid They have been exploited for fur since the Bering Expedition of 1741–42 yet until recently little has been known of their reproductive phenomena Barabas-Nikiforov (47) noted the ovarian shape Lensink (62) gave field observations on sea otter reproduction Sinha (65) described the morphology of the female genital tract based on 140 animals Sinha (65) gave a brief description of the ovarian morphology and Sinha Conaway and Kenyon (66) described its reproduction Pearson (52) described the ovarian and placental morphology from a single gravid decaying frozen female recovered from the beach at China Cove California It had a zonary chorio-allantoic placenta with an antimesometrial bilobed sac like hematome The placental structure described for the European river otter *Lutra vulgaris* by Bischoff (1865a) and for the American form *Lutra canadensis* by Creed and Biggers (64) seems to be essentially like that of the sea otter

The reviews of placentation in carnivores by Rau (25) Mossman (37) Amoroso ('52) and Starck (59) show that limited observations have been made on several carnivore genera but that the complete morphogenesis of the fetal membranes has been recorded only for the dog cat and ferret The description of the latter by Strahl and Ballmann (15) gives much useful detail on several stages from the blastocyst to full term Hamilton (34 and 37) made very careful studies of the period from fertilization to mesoblast and notochord formation More limited observations on mustelid placentation have been made on the ferret *Mustela furo* (Robinson 04 Lawn and Chiquoine 65) mink *Mustela vison* (Hansson 47 Enders 57) martens *Martes martes* and *foina* (Bischoff 1865b) weasel *Mustela vulgaris* (Bischoff 1865b) zorilla *Zorilla striata* (Rau 25) wolverine *Gulo luscus* (Wislocki and Amoroso 56) European badger *Meles meles* (Fleischmann 1891 Strahl 06 Amoroso 54) American

[1] This research wa supported by grant 86-5027 a Gen ral Re earch Support Grant to the Uni e sity of Wiscon in Medic l School from the N tion l Institutes of Health Division of Res rch Facilities and Resources Bethesda M ryl nd and in part by Pub l c Health Service grant HD 00277-09 The specimens for th tudy were gathe d under the supervision of M K rl W Kenyon in ooperati n with the Al ska Departm nt of Gam a part of th Bu eau f Sp rt Fi heries and Wildlife study of the life history of the sea otter

badger *Taxidea taxus* (Hamlett 32) and the skunks *Mephitis mephitis* and *macroura* and *Spilogale putorius* (Creed and Biggers 64)

It is apparent from the above literature that our knowledge of carnivore placentation and that of mustelids in particular is based upon relatively few species and usually upon a limited stage of gestation in a limited number of specimens Because nearly 40 pregnant sea otter uteri at most stages from blastocyst to very near term were available it seemed worthwhile to record the development of their fetal membranes in detail

## MATERIALS AND METHODS

The material consists of 39 gravid sea otter uteri collected around Amchitka Simeonof Little Koniuji Amak Shumagin, and the Aleutian Islands by Mr Karl W Kenyon of the Fish and Wildlife Service of the United States Department of Interior Most of the animals were killed by Fish and Wildlife Service personnel however a few were found dead on the beaches The tracts of the latter were often somewhat decomposed Reproductive tracts were excised and fixed in AFA (alcohol formalin and acetic acid) or 10% formalin and stored in 70% ethyl or isopropyl alcohol The material was made available to Dr Clinton H Conaway University of Missouri We are therefore greatly indebted to Mr Karl W Kenyon and Prof Clinton H Conaway

Macroscopic observations and measurements of fetuses placentae umbilical cords and hematomes were made on 19 animals (table 1) Appropriate pieces from these uteri were embedded cut at 6 to 10 μ and stained in Groat s tetrachrome stain Sample sections were also stained in periodic acid Schiff (PAS) Heidenhain s azan and hematoxylin and eosin Representative portions of hematomes were embedded in celloidin and stained in hematoxylin and eosin Twenty smaller gestation sacs were sectioned entire at 6 to 10 μ Ten of these were serially mounted where is only every tenth section of the others was mounted The same staining procedures were used as for the older specimens Unimplanted blastocysts were measured at their greatest diameter usi[ng] an ocular micrometer

## OBSERVATIONS

### Uterus and gestation sac

In sea otter uteri with unimplanted em[bryos] the endometrium usually has 4 to [6] asymmetrical rugae and is packed wi[th] coiled glands which form three distin[ct] zones the luminal compact zone the mi[d]dle spongy zone and the deep glandul[ar] zone (figs 14 15) However these zon[es] are greatly modified at the implantati[on] site so that each gestation sac has asym[]metrical endometrial lobes consisting [of] one major lobe and 3 to 6 minor ones Th[e] major lobe occupies the antimesometri[al] portion and extends a variable distanc[e] toward one or both sides of the horn Hi[s]tologically structures in both major an[d] minor lobes are alike The grooves or de[]pressions between the lobes are spanne[d] by the smooth chorion and the unmodifie[d] glands of their deep glandular zones ar[e] continuous with those of the deep glandular zones of the major and minor lobes The spongy zone is lacking at the groove (figs 16 17 19)

### Blastocyst

One unimplanted blastocyst was recovered from each of 11 sea otters Two of these were near implantation Most were shrunken and had broken zonae pellucidae but there were three normal oval ones with intact zonae These three range from 97 to 202 μ in diameter which suggests considerable growth before implantation Like most other mammals the sea otter blastocyst has a single layer of flattened trophoblastic cells closely adherent to the zona and an inner mass of cells (fig 13) Usually the zona pellucida is 4 to 6 μ in thickness and is composed of an outer thin more densely stained layer and inner thick homogeneous layer Blastocysts near implantation have approximately 500 cells in the inner cell mass Implantation is central (fig 1) essentially the entire circumference of the trophoblastic layer coming into contact with the uterine endometrium as in other carnivores (Mossman 37 and Amoroso 52)

TABLE

| Animal number | Total length of fetus in mm | CR length in mm | Chorio-allantoic band | Orientation of fetus in chorion | Orientation in gestation sac | Width of band in mm: Mesometrial | Lateral | At antimesometrial | Remnant of ...: Length | Width |
|---|---|---|---|---|---|---|---|---|---|---|
| 62–53 | 40 | 31 | complete | caudad | antimesometrial | 25 | 28/30 | 38 | — | — |
| 62–23 | 45 | 35 | incomplete | caudad | antimesometrial | 23 | 30/30 | 40 | 10 | — |
| 62–136 | 50 | 45 | complete | caudad | antimesometrial | 20 | 18/20 | 40 | 0 | — |
| 62–27 | 55 | 48 | incomplete | craniad | antimesometrial | 30 | 42/45 | 50 | 14 | 10 |
| 62–147 | 70 | 60 | complete | caudad | antimesometrial | 30 | 32/45 | 40 | 15 | 10 |
| 62–31 | 142 | 120 | incomplete | caudad | craniad | 35 | 38/40 | 57 | 55 | 25 |
| 62–29 | 190 | 160 | complete | caudad | craniad | 40 | 45/50 | 60 | 70 | 30 |
| 62–135 | 190 | 160 | incomplete | caudad | craniad | 50 | 65/65 | 80 | 100 | 35 |
| 62–8 | 225 | 190 | complete | craniad | craniad | 47 | 55/57 | 60 | 92 | 57 |
| 62–47 | 240 | 200 | complete | craniad | craniad | 53 | 67/65 | 72 | 84 | 65 |
| 62–109 | 280 | 230 | complete | craniad | craniad | 50 | 55/45 | 70 | 110 | 50 |
| 62–176 | 280 | 235 | complete | craniad | craniad | 55 | 70/70 | 80 | 75 | 40 |
| 62–33 | 320 | 270 | incomplete | caudad | caudad | 55 | 60/62 | 75 | 100 | 70 |
| 62–35 | 360 | 300 | complete | craniad | craniad | 55 | 80/80 | 85 | 105 | 15 |
| 62–37 | 3[illegible]0 | 315 | complete | craniad | caudad | 45 | 70/70 | 85 | 110 | 50 |
| 62–39 | 370 | 300 | complete | craniad | craniad | 60 | 70/75 | 85 | 70 | 70 |
| 60–4 | 3[illegible]0 | 300 | complete | craniad | craniad | — | — | — | — | — |
| 62–141 | 440 | 360 | incomplete | caudad | craniad | 0 | 65/75 | 85 | 165 | 60 |
| 62–119 | 440 | 370 | incomplete | caudad | lateral | 0 | 80/80 | 95 | 105 | 65 |

badger *Taxidea taxus* (Hamlett 32) and the skunks *Mephitis mephitis* and *macroura* and *Spilogale putorius* (Creed and Biggers 64)

It is apparent from the above literature that our knowledge of carnivore placentation and that of mustelids in particular is based upon relatively few species and usually upon a limited stage of gestation in a limited number of specimens Because nearly 40 pregnant sea otter uteri at most stages from blastocyst to very near term were available it seemed worthwhile to record the development of their fetal membranes in detail

## MATERIALS AND METHODS

The material consists of 39 gravid sea otter uteri collected around Amchitka Simeonof, Little Koniuji Amak Shumagin and the Aleutian Islands by Mr Karl W Kenyon of the Fish and Wildlife Service of the United States Department of Interior Most of the animals were killed by Fish and Wildlife Service personnel however a few were found dead on the beaches The tracts of the latter were often somewhat decomposed Reproductive tracts were excised and fixed in AFA (alcohol, formalin and acetic acid) or 10% formalin and stored in 70% ethyl or isopropyl alcohol The material was made available to Dr Clinton H Conaway University of Missouri We are therefore greatly indebted to Mr Karl W Kenyon and Prof Clinton H Conaway

Macroscopic observations and measurements of fetuses placentae umbilical cords and hematomes were made on 19 animals (table 1) Appropriate pieces from these uteri were embedded cut at 6 to 10 μ and stained in Groat s tetrachrome stain Sample sections were also stained in periodic acid Schiff (PAS) Heidenhain s azan and hematoxylin and eosin Representative portions of hematomes were embedded in celloidin and stained in hematoxylin and eosin Twenty smaller gestation sacs were sectioned entire at 6 to 10 μ Ten of these were serially mounted whereas only every tenth section of the others was mounted The same staining procedures were used as for the older specimens Unimplanted blastocysts were measured at their greatest diameter usi an ocular micrometer

## OBSERVATIONS

### *Uterus and gestation sac*

In sea otter uteri with unimplanted e bryos the endometrium usually has 4 t asymmetrical rugae and is packed w coiled glands which form three distu zones the luminal compact zone the m dle spongy zone and the deep glandu zone (figs 14 15) However these zor are greatly modified at the implantati site so that each gestation sac has asy metrical endometrial lobes consisting one major lobe and 3 to 6 minor ones T major lobe occupies the antimesometr portion and extends a variable distan toward one or both sides of the horn H tologically structures in both major a minor lobes are alike The grooves or c pressions between the lobes are spann by the smooth chorion and the unmodifi glands of their deep glandular zones a continuous with those of the deep gland lar zones of the major and minor lobe The spongy zone is lacking at the groov (figs 16 17 19)

### *Blastocyst*

One unimplanted blastocyst was r covered from each of 11 sea otters Tw of these were near implantation Mo were shrunken and had broken zonae pe lucidae but there were three normal ov ones with intact zonae These three rang from 97 to 202 μ in diameter which su gests considerable growth before implant tion Like most other mammals the se otter blastocyst has a single layer of fla tened trophoblastic cells closely adheren to the zona and an inner mass of cell (fig 13) Usually the zona pellucida i 4 to 6 μ in thickness and is composed o an outer thin more densely stained laye and inner thick homogeneous layer Blas tocysts near implantation have approxi mately 500 cells in the inner cell mass Implantation is central (fig 1) essen tially the entire circumference of the trophoblastic layer coming into contact with the uterine endometrium as in other carnivores (Mossman 37 and Amoroso 52)

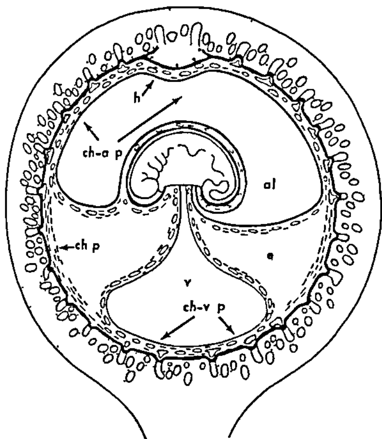



Fig. 2 Diagram of a cross section of the uterus at a very early stage of attachment showing allantoic vesicle al exocoelom e vitelline cavity v chorionic placenta ch p chorio-allantoic placenta ch a p chorio-vitelline placenta ch v p hematome h

*Nonvascular chorionic placenta* The area of nonvascular chorionic placenta tion in the sea otter depends upon the extent of exocoelom formation in the area vasculosa. It is located on the lateral sides of the gestation sac (figs 2 16) and appears to be a functional portion of the placental mechanism as in the cat (Amoroso 52)

*Chorio-allantoic placenta* The chorio-allantoic placenta of the sea otter is typical of that of other carnivores Its development starts just before the limb buds appear but the zonary band is completed somewhat later The allantoic vesicle is large (fig 16) Except for its antimesometrial location and vascularization by allantoic vessels the chorio-allantoic placenta (fig 22) at this stage is strikingly similar to the chorio-vitelline placenta (fig 21) It is from the first endotheliochorial Distinct giant cells are absent as in the zorilla ferret and dog

### *Fetal membranes from the early fetal period to term*

Once the chorio-allantoic placenta is established further changes are essentially elaboration of the pre-existing structures At each of the stages described below the placenta consists of a typical zonary band which may be complete or incomplete because of a narrow gap mesometrially Each annulus is narrower in width mesometrially than antimesometrially and has 4 to 8 lobes of different size (fig 9) The hematome is located antimesometrially on the zonary band It will be described separately The fetus may face either cranially or caudally in the uterus and there

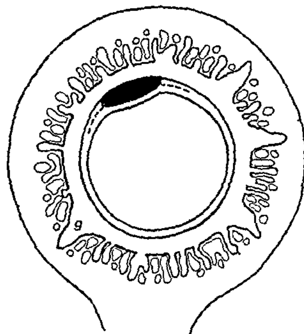



Fig 1 Diagram of a cross section of the uterus showing the central location of the blastocyst and the relatively antimesometrial orientation of the embryonic disc Asymmetrically located grooves g divide the endometrium into irregular lobes Mesoderm dashed lines

*Early fetal membranes through the limb bud stage*

Only one embryo of this group of nine has nonvascular bilaminar omphalopleure The other eight have three distinct regions in the gestation sac the chorio-vitelline placenta the nonvascular chorionic placenta and the chorio allantoic placenta (figs 2 16) The relative size and position of these placental areas varies from animal to animal Each embryo is antimesometrial but somewhat lateral to the midline Placental differentiation and relative embryo growth are probably rapid after implantation begins especially up through the limb bud period

*Bilaminar omphalopleure* In the one sea otter with a nonvascular bilaminar omphalopleure the trophoblastic villi are straight and solid (fig 20) They are mostly cellular but a syncytial mantle is frequently observed on those villi penetrating the glandular symplasma and blocking the lumina of hypertrophied spongy zone glands Occasionally a villus has a mesodermal core and is branched Glands usually have cuboidal cells with centrally located nuclei However those glandular cells near the villi hypertrophy to form columnar cells which often fragment and become pycnotic followed by shrinkage and symplasma formation

*Chorio-vitelline placenta* In eight specimens the area of chorio vitelline placentation has been restricted by the extension of the exocoelom into the area vasculosa thus splitting the chorio-vitelline placenta to form two new structures the outer nonvascular chorionic placenta and the inner free splanchnopleuric yolk sac wall (fig 16) The chorio vitelline placenta becomes more and more restricted on the mesometrial side however, it is better developed than in the cat and dog (Amoroso 52) Although less massive and elaborate than the chorio-allantoic placenta it has the same regions the labyrinth, junctional zone and the spongy and deep glandular zones

The chorio vitelline placental labyrinth is established just before the limb buds appear, and persists through the limb bud period No hematome is formed in the region of the chorio vitelline placenta The labyrinth occupies roughly a third of the placental depth (figs 19 21) The trophoblastic villi have mesodermal cores and a few very delicate fetal capillaries The villi are branched and usually tortuous and form networks enclosing maternal capillaries The maternal capillary endothelium is thickened as in the ferret mink wolverine and raccoon but unlike that of the dog and cat which is more squamous Its PAS positive and diastase resistant basement membrane is relatively thin Thus an endotheliochorial condition is well established in this chorio vitelline placenta

Villi usually occupy the glands in the junctional zone and the adjacent portion of the spongy glandular zone They apparently absorb histiotrophic material The spongy zone glands are hypertrophied greatly dilated and irregularly folded and branched Their adjacent walls form septa (figs 19 20) Each septum is bounded on either side by cuboidal or low columnar epithelial cells which enclose its core of maternal connective tissue and vessels Maternal vessels enter and leave the labyrinth through these septa In the deep glandular zone the glands are unmodified and their bases reach the myometrium

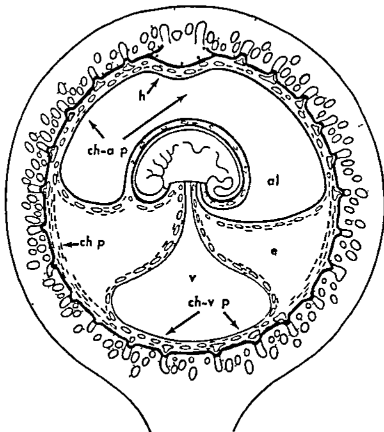



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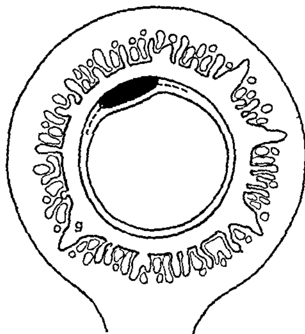



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*Definitive yolk sac*

The splanchnopleuric yolk sac of the sea otter is a large permanent structure as in other carnivores (Mossman 37) and is restricted to the mesometrial side (fig 3) The sac is elongated in the direction of the uterine axis and is free except for a threadlike mesodermal attachment at each of these ends It remains vascularized throughout gestation

*Hematome*

Placental hematomes are characteristic of many carnivores and of a few insectivores (Strauss 43) and bats (Wimsatt and Gopalakrishna 58) They consist of a mass of extravasated maternal blood lying between the vascular chorio-allantoic membrane lined by phagocytic cytotrophoblast and a glandular area of the endometrium The accumulation of blood may be

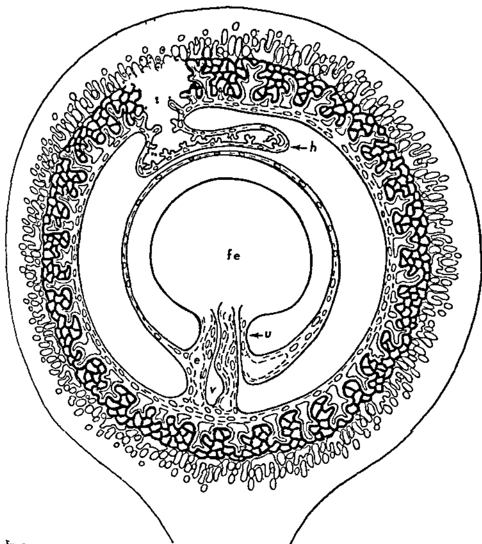



Fig 3 Diagram of a cross section of the uterus in late pregnancy The chorio-allantoi placenta is now annular The chorio-vitelline placenta has disappeared Fetus fe umbilical cord u other abbreviations as in figure 2

is no correlation between its orientation and the position of the hematome (table 1)

*Intermediate stage of the chorio allantoic placenta* In the five animals having fetuses between 40 and 70 mm in length the placental labyrinth occupies roughly two-thirds of the placental depth (figs 23 25) In stained sections it appears less compact than the labyrinth of the mature placenta (fig 28) It has a series of lamellate fetal villi between which are the branched and tortuous maternal capillaries intimately enclosed by the syntrophoblast (fig 24) Instances of intratrophoblastic fetal capillaries are frequent thus maternal and fetal blood are brought into greater proximity Basement membranes of the maternal capillaries are relatively thinner near the junctional zone than at the fetal surface This indicates that the increase in the thickness of the basement membrane is a later acquisition probably produced by the deposition of argyrophilic fibers of maternal endothelial cell origin as in the zorilla (Rau 25) and wolverine (Wislocki and Amoroso 56) This difference in the thickness of basement membrane is not noticeable in the mature placenta

Most of the villi have not yet penetrated beyond the central ends of the spongy zone glands however a few have entered deeper into their lumina (figs 23 25) Those portions of the villi which project into the lumina have tall columnar phagocytic cytotrophoblastic cells with more or less basal nuclei and usually are devoid of syncytial cells The glands are more dilated hence the septa are thin at this stage Usually the basal ends of the dilated portions are smooth (figs 23 25)

At the margin of the placenta the zonary band is deeply undercut (fig 25) by a fold of chorio allantois The annulus gradually thins towards its margins The folds in the undercut margin have tall columnar cytotrophoblastic cells with more or less basal nuclei (fig 40) These cells are phagocytic and apparently absorb histiotrophic material from the partially degenerate spongy zone glands of the area Normally there is no extravasated blood in the marginal phagocytic region contrary to the condition in the dog where the marginal region is a hematome

Beyond the placental margin the chorio-allantois and the uterine epithelium are separated only by a thin space usually under 1 mm in thickness which contains variable amounts of histiotrophic material The chorio allantois is a smooth membrane whose cuboidal trophoblastic cells appear to be absorptive The uterine epithelium is also cuboidal (fig 40)

*Definitive chorio allantoic placenta* In the 14 animals having 142 to 440 mm fetuses the placenta has reached its definitive structure but does of course increase in size during this period The labyrinth occupies roughly four fifths of the placental depth (fig 28) It is still endotheliochorial however the complexity of the maternal and fetal capillaries has greatly increased and instances of intratrophoblastic fetal capillaries are much more numerous (fig 42) By light microscopy these appear to be apposed to the basement membrane of the maternal endothelium (fig 42) Thus maternal and fetal blood are in even greater proximity and if the trophoblast has actually disappeared from these areas they would be endothelio endothelial in nature The fetal capillaries usually have flattened endothelial cells and thin PAS positive basement membranes

Both the junctional and spongy zones are greatly compressed and the villi penetrate into the spongy gland lumina as in the raccoon (Creed and Biggers 63) These villi are often branched inside the lumina Instead of syncytium their intraglandular portions are covered by tall columnar phagocytic cytotrophoblastic cells with basal nuclei (fig 34) These are obviously histiotrophic absorptive areas and their trophoblast is very similar to that lining the hematome The deep glandular zone is greatly reduced

*Amnion*

During early pregnancy the amnion is a thin membrane separated from the allantois by the voluminous extra-embryonic coelom but by the time the embryo has reached about 70 mm the amnion is well vascularized by the allantoic vessels except for a small area adjacent to the umbili cord (figs 2 3)

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Fig 4 Early labyrinth and hematome pouches

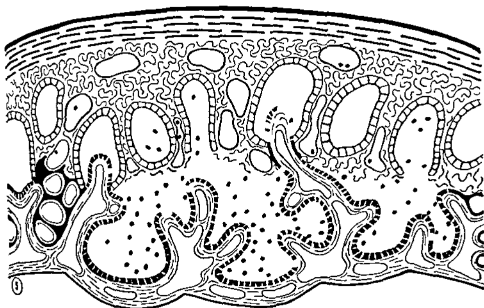


Fig 5 Coalescence of the pouches initiates the formation of a central major hematome pouch

so massive that the chorio allantois is bulged inward to form one or more large sacs which are sometimes pedunculated In such cases the blood appears at first sight, to be entirely enclosed by the chorio allantois because the only contact with endometrium is the relatively small area at the base of the stalk (or stalks in the case of multiple sacs) At the other extreme are absorptive areas ranging in size from relatively large to microscopic in which there is no grossly visible extravasate yet in which microscopical examination shows active phagocytosis of maternal erythro cytes by the lining cytotrophoblast In some species there is little or no erosion of the epithelium of the basal endome trium, but in others the septal ridges be tween the glands lose all or most of their epithelium as in the sea otter In the definitive larger hematomes the inner vas cular layer is almost always elaborately folded or villous or both Its cytotropho blastic lining is columnar and obviously absorptive and phagocytic Where pla cental labyrinth borders a hematome a single chorio-allantoic fold or villus may serve the hematome on one side and the labyrinth on the other side (fig 39) Be sides being areas for contact with and absorption of maternal blood hematomes contain at least some glandular secretion and therefore are functionally both "he motrophic and histiotrophic" Struc turally they are homologous to such absorp tive areas as chorionic vesicles areolae, and rosettes known in the placentae of many mammalian groups including the carnivores themselves moles (Talpidae) lemurs galagos lorises perissodactyls and artiodactyls (Mossman 37 Amoroso 52)

Placental absorptive areas including he matomes are clearly significant structures both from the standpoint of the light they may throw on the phylogenetic develop ment of the placenta and the interrela tionships of placental mammals and also from that of placental function Unfortu nately we still know very little for certain about either of these aspects Are they primitive or specialized placental mech nisms? Is the contained blood mass of a hematome truly an extravasate or does it slowly circulate through the hematome? What brings about the extravasation? Does some form of digestion of the blood and glandular secretions take place in the hematome as Creed and Biggers (63 suggested? Are the red blood cells phago cytosed entire and still alive" or are they dead or fragmented? What is the nature of the lining trophoblast cells which al lows them to function in these ways and how and in what form are the absorbed substances transferred to the fetal blood? What is the developmental history of a placental hematome?

Our sea otter material can contribute very little toward the solution of most of these problems but we have been able to follow some features of the development of the hematome from its first appearance at the limb bud stage to term

*Early development of the hematome* The hematome first appears during the limb bud period on the antimesometrial side of the chorio allantoic placenta usu ally on the major endometrial lobe and somewhat laterally (figs 2 18) This is at the time when the early vascular chorio-allantoic villi have begun to pene trate into the mouths of the endometrial glands and when labyrinth in beginning to form between them through the erosion of the epithelium covering the intergland ular area followed by engulfment of the maternal capillaries by the syntrophoblast In the area of the hematome materna blood appear as an extravasate between the villi It is not clear in our materia whether the maternal vascular rupture is brought about by syntrophoblastic erosion or whether syntrophoblast is actually lack ing at these points The endometrial sur face epithelium between the gland mouths is missing in the areas where the first ex travasations appear and the villi extend into the gland necks but blood first ap pears between the villi and the gland walls

Figs 4–8 Diagrams of vertical sections through the antimesometrial area of the chorio allantoi placenta and uterine wall to show the develop ment of the labyrinth l and hematome h Uterine serosa se myometrium my endome trial stroma s gland epithelium gl materna blood m early hematome pouches h p syn trophoblast (heavy black) sy phagocytic cyto trophoblast (heavy black cross hatched) cy fetal blood vessels f allantoic mesenchyme me allantoic endoderm en

chorio-allantoic membrane between the villi is bulged inward toward the allantoic cavity (figs 4-8) The villi become progressively detached from the endometrium allowing enlargement of the pouches As this process continues smaller pouches are incorporated into their larger neighbors while at the same time newer ones may be forming peripheral to the earlier ones (figs 4 5 6 29 30) All this is happening when the whole hematome may be only 1 to 5 mm in diameter However this process probably stops rather early as where labyrinth has once formed it is difficult to see how new hematomes could develop and also the hematomes of later stages although as wide or wider than the zonary labyrinth always have a narrow peduncular base 5 to 10 mm in diameter (figs 10 11 31 32)

In each pouch the cytotrophoblast becomes a tall columnar epithelium with basal nuclei (fig 37) The cytoplasm of many of these cells is soon filled with

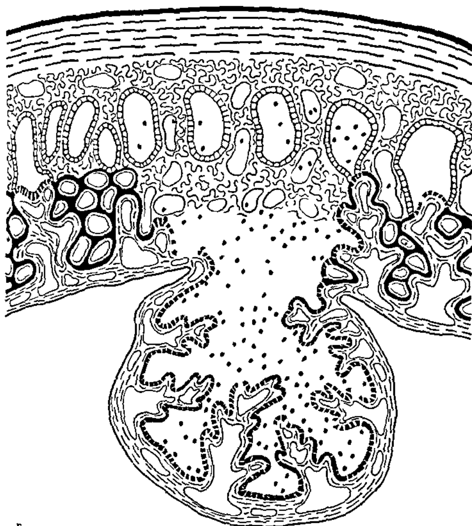


Fig 7 Beginning formation of the hematome stalk The basal fibrous zone is shown as it appears in a section slightly lateral to the central axis of the stalk Connections of the gland lumina to the stalk cavity are not shown because they are present only at the center of the base of the stalk

not deep in the glands (figs 4 5) The *trophoblast lining these areas is high columnar and phagocytic* If syntrophoblast is initially present, it must separate completely from the cellular layer but since none is identifiable on the endometrial side when the plane of section is vertical *through the base of the hematome* we think it either disappears very early or has always been absent from these potential hematome areas Neither does our material tell us for certain whether extravasation takes place by leakage from ruptured capillaries or by *diapedesis* However, two observations make diapedesis seem unlikely first there is no infiltration of erythrocytes into the stroma as one would expect unless diapedesis were confined strictly to those vessels directly bordering the potential hematome space and second the massive amount of blood that soon accumulates in the hematome as well as the pressure *which it must exert to produce the bulging sac like shape* suggests leakage open vessels No open vessels have been observed but the capillaries of the fibrous capsular area are shrunken and have degenerate endothelial cells (fig 35)

*In the sea otter the first extravasations* are near one another but not necessarily contiguous As blood accumulates the

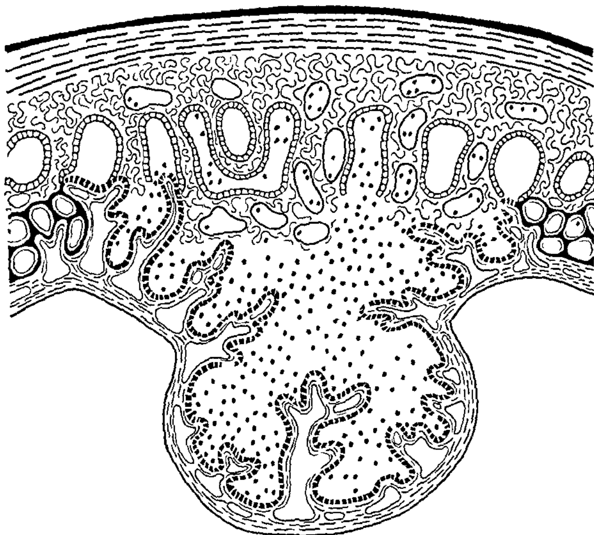


Fig 6 Increasing extravasation of maternal blood and additional coalescence of immediately adjacent pouches results in a prominent central hematomal sac Much of its base consists of fibrous endometrial stroma with many maternal capillaries but in a few places underlying gland lumina are continuous with the hematomal blood spaces and are themselves filled with maternal blood

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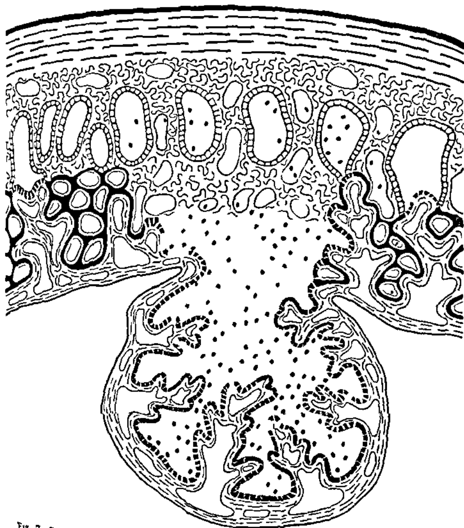


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phagocytosed maternal erythrocytes De generating multinucleate masses presumably of epithelial origin but possibly syntrophoblastic are often seen in the contents of the pouches (cf Wimsatt and Gopalakrishna 58) As the pouches increase in size, numerous large complexly branched lamellate and villous folds differentiate and subdivide the blood masses in their lumina

At the time the hematomal pouche first form the underlying endometriur is like that already described for the lab rinthine area but when the pouches begi to coalesce changes occur especially i the junctional zone Instead of being ir vaded by syntrophoblast to form labyrint the stroma becomes more fibrous the c pillaries become narrower their epithe lium shows degenerative changes an

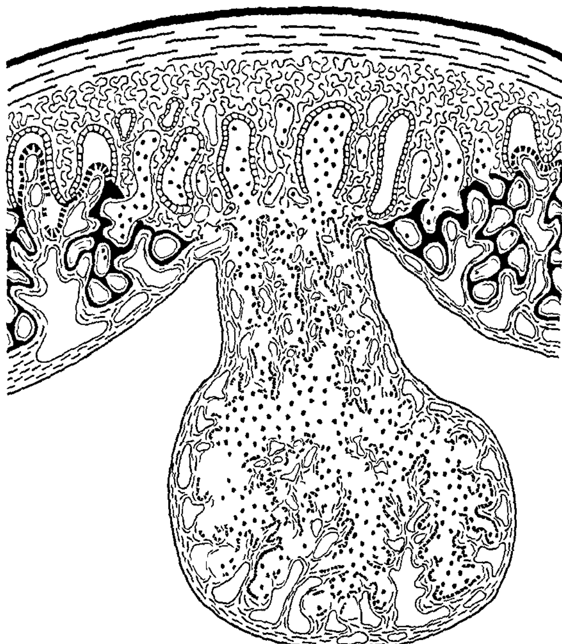


Fig 8 This shows the basic structure of the definitive chorio allantoic placental labyrinth and the hematome i e the numerous richly vascular laminae and villi of the major sac and stalk the maternal blood in the dilated basal glands the endotheliochorial labyrinth and the phagocytic cytotrophoblast covering the tips of the allantoic villi which occupy the open ends of the basal glands

their diastase resistant basement membranes become thicker and more strongly PAS positive (fig 35) This somewhat fibrous capsule like zone separates the hematomic pouches from the dilated spongy gland zone (figs 7 8 32 35) except for central perforations left by the necks of the glands It is continuous at its margins with the surrounding labyrinth where of course syntrophoblast becomes conspicuous Leakage from maternal vessels of this zone seems to be the most likely source of the blood filling the hematome Deeper in the septa of the spongy zone the maternal capillaries are normal

*Definitive hematome* From the early fetal period onward the hematome increases markedly in size and complexity but does not change otherwise except that the central portion becomes more and more prominent and definitely stalked while the peripheral pouches become less prominent and tend to disappear presumably by incorporation into one another or into the main one Unlike that of the mink and raccoon the hematome maintains its large size to term (table 1) As shown in figures 3 7 8 32 33 it invaginates the chorioallantois toward the allantoic cavity and so is covered to the base of its stalk by the allantoic endoderm It is so large and so closely pressed against the amnion and fetus that in preserved specimens each bears imprints of the other (figs 10 11 12) No doubt in the living the hematome is a semi turgid sac but because of the variety of positions it may take with reference to the fetus the preserved organ may be compressed oval pear shaped saucer like or ball shaped (figs 10 11 12) It is composed of one or two large central lobes attached to the junctional zone by a long narrow stalk There are several small accessory pouches at its base In one animal (62–39) both the hematome and placental annulus were cephalic to the fetus which had an umbilical cord about 70 mm long instead of the normal 10 to 30 mm In this case the body of the hematome was ball shaped

Microscopically there are three regions of the main central lobe of the definitive hematome the body the stalk and the junctional and spongy zone or base (fig 8) The body is a thick walled vascular chorio-allantoic sac much like that of the wolverine badger and raccoon It contains 75 to 95% of the extravasated blood It is covered by low cuboidal or squamous allantoic endoderm and is lined by tall phagocytic and absorptive cytotrophoblast Numerous profusely branched villus like folds divide the main sac into smaller yet confluent compartments (figs 8 32 33) These folds actually appear to take up

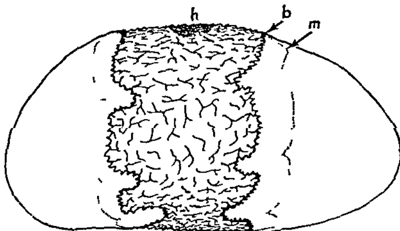



Fig 9 Diagram of a whole conceptus removed from the uterus to show the lobed zonary band and the area of attachment (internally) of the hematome h on the antimesometrial portion of the zone Free margin of annular placenta m border of basal attached portion b

almost as much space as the maternal blood does The folds are fewer and simpler near the stalk They are highly vascular and their capillaries have distinct basement membranes The distal cytoplasm of most of the cytotrophoblast cells is packed with maternal erythrocytes or fragments of them and with orange red crystals No doubt other materials originating from the blood or glandular secretions are phagocytosed but our material was unsuitable for identification of such elements The intensity of pigmentation of the extravasated blood still occupying the hematome at full term suggests that the trophoblast rejects large amounts of blood pigment while it presumably selects and absorbs the iron

The stalk is distinctly demarcated from the placental labyrinth by a deep groove lined by the chorio allantoic (figs 7 8 32 33 34) Its structure like that of the main body except that th vascular folds clearly parallel its long axis and thus divide it into 15 to 30 narrow channels through which the materna blood enters the body of the organ These folds are the pathways for the fetal vessels supplying the entire sac and are continuous with those of the main body They are also covered by phagocytic epithelium (figs 36 39)

The junctional and spongy zone glands at the base of the definitive hematome are much more complex than earlier (figs 31 32) The lumina are filled with ma




Fig 10 Gestation sac with fetus (total length 115 mm) with one side of gestation sac and membranes removed to show the major sac of the hematome with several smaller basal pouches Note the narrow stalk of the major sac Note also the narrowness of the mesometrial portion of the zonary placental band compared to the antimesometrial portion (above in the fig ) (Photo by K W Kenyon of JEB 63–40 which is not included in our tables )



Fig 11 Gestation sac with fetus (total length 118 mm) showing the very large stalked hematome The placenta is cut almost at the center of the hematome stalk The mesometrial portion of the placental band (below in fig ) is relatively broad in this specimen (Photo by K W Kenyon of JEB 63-83 which is not included in our tables )

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DISCUSSION

There is paleontological evidence of a close phylogenetic relationship between the sea otter and the river otter (Taylor 14) Relatively little is known of the fetal membranes of the river otters *Lutra vulgaris* (Bischoff 1865a) and *Lutra canadensis* (Creed and Biggers 64) but grossly they are very similar to those of *Enhydra* All three have somewhat lobate usually completely zonary endotheliochorial placentae with a large bag like antimesometrial hematome attached by a stalk surrounded by smaller hematomal pouches

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Fig 12 Gestation sac with fetus (total length 186 mm) showing lobation of zonary band (upper portion in fig ) and the large hematomal pouch dorsal to the forward half of the fetus (Photo by K W Kenyon of ADFG 520 which is not included in our tables )

mens of the mink *Mustela vison* and long tailed weasel *Mustela frenata* all in the mid to late fetal period also have incomplete zonary placentae with anti mesometrial non stalked hematomes The skunks *Mephitis mephitis Mephitis macroura* and *Spilogale putorius* based on one uterus each with embryos of 32 35 and 7 mm respectively all have complete zonary placentae with broad antimesometrial hematomes (Creed and Biggers 64) Whether or not some or all of these American mustelids arrive at a double discoid shape or some other variation in gross placental form late in gestation is uncertain Such changes in form would not be unlikely due to rapid increase in uterine girth possibly accompanied by pressure atrophy of labyrinth adjacent to the bulging hematome which is presumably turgid with extravasated maternal blood

From the above it is clear that closely related mustelids do have placentae of similar external form although their form

may change much during development It might therefore be argued that gross placental characters indicate closer relationship between *Enhydra Lutra* and *Gulo* than with the other mustelids studied This may be true but one must remember that Creed and Biggers (63) have shown that the placenta of the raccoon *Procyon lotor* a member of the family Procyonidae also has an irregular zonary shape with a large stalked antimesometrial hematome Furthermore the dog and sea lion members of different suborders have zonary placentae which differ from each other only in very minor details So while it is generally true that members of the same genus or even of the same family have like placental shapes the converse that placentae of the same shape within such a group indicate close relationship is not a safe deduction (For further discussion of the fetal membranes and phylogeny see Mossman 37 53 Amoroso 59)

One encounters an equally confusing and even more speculative set of concepts when one seeks to trace the evolution of a specific placental characteristic or type within the mammalian group Rau (25) for example attempted to show the origin in carnivores of the double discoid placenta with an isolated hematome such as that of the mustelids from the zonary placenta with the more extensive yet less complex green border hematome such as that of the canids He placed much weight on the fact that some mustelid placentae start out as zonary but later become double discoid Rau was handicapped by incomplete information especially regarding raccoon placentation Considerably more is now known about carnivore placentation but far too little to allow one to produce convincing evidence of its evolutionary course We are however inclined to a view somewhat opposite to that of Rau partly because the mustelids procyonids and ursids are considered to be relatively primitive groups and the canids felids and pinnipeds relatively specialized Also it seems reasonable to us to assume that the first mammalian placentation would have been epitheliochorial and diffuse (i e broadly zonary) However when more intimate relationships between the endometrium and chorio-allantois developed there is no reason to assume that it involved the whole zonary area Since extravasation of maternal blood is a common occurrence in the endometrium during and even before implantation an easily imagined first step in supplementation of the epitheliochorial and uterine secretion stage of fetal nutrition would be formation of hematomatous masses of noncirculating maternal blood confined between the endometrium and the vascular absorptive chorio-allantois Phagocytosis of extravasated maternal blood along with absorption of glandular secretions would seem a far simpler mechanism to evolve than a labyrinth with circulating maternal blood whether of the hemochorial or endotheliochorial type Also placentation of this sort could easily be adequate for short gestation periods with young being born as most marsupials are in a very immature state As intrauterine development was carried further there would have been more and more need for the elaboration of a structure permitting rapid and efficient interchange especially of respiratory gases and it is in response to this need that zones of intimate contact between the fetal and maternal capillaries would have evolved We are therefore inclined to think that isolated hematomes may be as old or older phylogenetically than labyrinths Although hematomes and labyrinths are both formed primarily of chorioallantois we think it likely that they arose independently in time Perhaps the best argument against our view is the fact that hematomes are relatively rare as such in mammalian placentae and in fact are unknown in the placentation of marsupials and reptiles where simple labyrinths have however been evolved Yet one must recognize that absorptive areas areolae and the like are common in mammalian placentae and areas functioning in a similar fashion do no doubt exist in marsupials and reptiles and since we believe that hematomes are essentially specialized areolae or chorionic vesicles the absence of blood in absorptive areas may not be very significant

Such speculations as Rau s and our own are probably of most value in drawing

attention to the paucity of information with which all of us are working It is remarkable that the paper by Strahl and Ballmann on the ferret and this one on the sea otter are the only ones attempting to describe the full developmental history of the fetal membranes of a mustelid a group represented by at least 29 recognized living genera In fact of the well over 100 genera of Carnivora the only others for which the morphogenesis of the fetal membranes has been adequately described are the domestic dog and cat At present even granting an enormously greater knowledge of comparative placentology, it is difficult to believe that such questions of placental evolution can ever be answered with complete scientific certainty However future advances in our understanding of the mechanisms of heredity and differentiation may give us insight far beyond our present capacity

The ultrastructure and specific function of hematomes and other phagocytic absorptive areas are also matters of uncertainty There is the somewhat academic question as to whether a hematome is an example of epitheliochorial or hemochorial placentation In the dog and especially in the cat where accumulation of extravasated maternal blood is minimal the blood clearly lies between relatively intact *uterine epithelium and the phagocytic* trophoblast of the chorio allantois Such a hematome is an area of the chorio allantois which is designed to absorb uterine contents composed of glandular secretions ( uterine milk ) *and* extravasated maternal blood It is obviously homologous structurally with the absorptive areas or areolae well known in the pig placenta but also present in the moles lemurs galagos lorises cetaceans perissodactyls non ruminant artiodactyls and camels The placentae of all of these are largely epitheliochorial and the absorptive areas can also be considered epitheliochorial as Wislocki and Padykula ( 61) did But what of the large stalked hematome characteristic of the sea otter and raccoon (Creed and Biggers 63)? Here a large mass of maternal blood is enclosed in a complexly partitioned bag of absorptive vascular chorio allantois and the only connection with the uterine epithelium is at the narrow base of the stalk r maternal blood is so characteristically contact with trophoblast while maternal epithelium occurs only at the base of the stalk and because there is also some sion of uterine epithelium between glands Creed and Biggers consider the raccoon hemophagous organ a form of hemochorial placentation Strictly on the basis of the criteria used one can make a case for interpreting placental hematomes as either epitheliochorial or hemochorial However Grosser applied his system of classification *to the zone of the chorio* allantoic and chorio vitelline placentae characterized by *fetal capillaries in close apposition to channels containing actively circulating maternal blood* to what might be called the zone of double circulation or vascular intimacy in other words the labyrinthine zone or the villous zone This application of the Grosser system of terminology has been generally but not universally followed To us it seems best to continue this application In that case since the maternal blood of a hematome is almost certainly not circulating and since it occupies a space along with uterine histiotroph we prefer to regard such structures as supplementary placentae if they are large and clearly demarcated and simply as phagocytic absorptive areas *if they are small* and scattered in close association with the villous or labyrinthine zones Enough confusion exists in the application of the still very useful Grosser terminology when *one confines it* to the areas of dual circulation or *zones of intimacy* of chorio allantoic and chorio vitelline placentae The larger absorptive areas in carnivores almost always contain maternal blood and can be adequately distinguished by the term placental hematome or hemophagous organ Nothing is gained by applying the Grosser terminology to them as they are all essentially alike In fact the histiotrophic absorptive areas of the carnivores and the many other groups mentioned above are of the same fundamental structure as the hematomes and hence do not need specific designation by Grosser s terminology

The problem of hematome function is well discussed by Creed and Biggers (63) yet somewhat futilely because of the lack

of experimental evidence It is certain that the placental hematome unlike the pathological one is a functional organ Wislocki and Dempsey (46a b) clearly demonstrated the absorption of iron by the columnar cytotrophoblastic cells of the hematome and junctional zones of the cat and dog and by the areolae and chorionic vesicles of the pig The highly developed columnar trophoblastic cells are clearly specialized for phagocytic purposes Yet the exact manner of utilization of the blood is unknown No doubt many elements of the plasma are also absorbed in conventional manner but even this awaits proof Certainly there is as yet no evidence of active digestive processes in absorptive areas but there is no doubt that in some cases whole erythrocytes are phagocytosed by the trophoblastic cells and that in others there is much breakdown of corpuscles and of hemoglobin itself in the cavity of the hematome The green pigment in the dog hematome and similar rich pigmentation in hematomes of other species is direct proof of this cellular and chemical breakdown Observation of apparently entire erythrocytes in the cytoplasm of the lining trophoblast cells in many forms including the sea otter black bear dog cat and bobcat (*Lynx rufa*) is good evidence for phagocytosis before erythrocyte breakdown Apparently both processes usually occur together but perhaps in certain species only one prevails For instance cats including the bobcat show relatively little pigmentary or other gross evidence of a marginal hematome yet sections of the area disclose large numbers of maternal erythrocytes being phagocytosed by the trophoblast lining a zone entirely comparable otherwise to the dog's green border Apparently in such cases phagocytosis occurs as fast as extravasation hence there is little accumulation of maternal blood and most hemoglobin breakdown must occur intracellularly after phagocytosis and possibly during further transport to the fetal blood stream In such hematomes as those of the mustelids the black bear and dog extravasation rate exceeds phagocytosis rate and therefore large masses of blood accumulate with resulting large scale hemolysis and hemoglobin breakdown in the hematome On the face of it this latter situation seems less efficient and well adapted than the situation in the cats This accords with our idea that the placentae of mustelids are relatively primitive and those of the felids more advanced

The similarity of the fetal membranes of *Enhydra* to those of other mustelids is another example of the relative conservatism of fetal membrane characters within a major mammalian group of fairly certain phylogenetic interrelationships Although the series of ontogenetic stages of the sea otter membranes is not as closely graded as could be desired it is complete enough to demonstrate that there is no significant variation from that of the ferret dog or cat the only others as well known In fact we have noted such complete similarity to these others in character of blastocyst yolk sac amnion allantoic vesicle and umbilical cord that it has been unnecessary to describe these features other than to point out that they are typical of carnivore membranes so far studied It seems probable in view of the known similarities between the later stages of the fetal membranes of representatives of the pinnipeds and of the seven families of fissipeds that there are no major divergences of membrane type within the order Carnivora although the detailed morphology of the hyaena placenta is certainly somewhat unusual (Morton 57 Wynn and Amoroso 64) and more information is needed to understand just how far it differs from the type If differences of fundamental nature are found they will probably be in the most primitive or the most specialized or aberrant genera

*Synopsis of basic data on the fetal membranes of the sea otter*

Implantation
Orientation (disc) Antimesometrial
Orientation (first attachment) Antimesometrial and mesometrial becoming zonary
Depth Superficial
Decidua None Great hypertrophy and dilation of uterine glands
Amniogenesis Folding
Chorion Present early but all eventually converted to chorio-allantois

Yolk sac

Bilaminar omphalopleure Temporary

Chorio-vitelline placenta Well developed and persists through limb bud stage

Vascular splanchnopleure Medium sized collapsed permanent bilobed or T shaped sac attached by a strand at each arm of the T to the chorion

Chorio-allantoic placenta

Shape Annular lobed (sometimes incomplete) Type Labyrinthine

Finer morphology Endotheliochorial (appears by light microscopy to be endothelio-endothelial at many points late in gestation)

Accessory placentae Antimesometrial pedunculated sac like hematome surrounded by smaller hematomic pouches

Location Annular

Allantoic vesicle Large and permanent

Authorities Authors

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Yolk sac
  Bilaminar omphalopleure Temporary
  Chorio-vitelline placenta Well developed and persists through limb bud stage
  Vascular splanchnopleure Medium sized collapsed permanent bilobed or T shaped sac attached by a strand at each arm of the T to the chorion
Chorio-allantoic placenta
  Shape Annular lobed (sometimes incomplete) Type Labyrinthine
  Finer morphology Endotheliochorial (appears by light microscopy to be endothelio endothelial at many points late in gestation)
  Accessory placentae Antimesometrial pedunculated sac like hematome surrounded by smaller hematomic pouches
  Location Annular
Allantoic vesicle Large and permanent
Authorities Authors

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## PLATE 1

### EXPLANATION OF FIGURES

13 Unimplanted blastocyst in the uterine lumen The irregular scattering of the cells is probably an artifact Note the large number of uterine glands characteristic of carnivores 107–55 × 80

14 Cross section of uterus showing asymmetrical endometrial lobes and large collapsed blastocyst probably near time of implantation 62–41 × 11

15 Higher magnification of uterine wall of figure 14 showing the luminal compact zone the still thin middle spongy zone where the gland lumina are just beginning to dilate and the relatively thick deep glandular zone 62–41 × 52

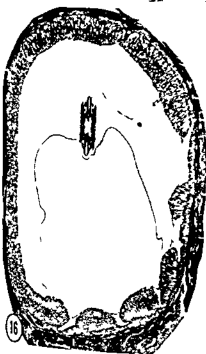
16 Cross section of gestation sac showing endometrial lobes and grooves spanned by smooth chorion Note the lack of spongy zone at the grooves Between the antimesometrial allantoic sac (above in fig ) and the mesometrial yolk sac is the large exocoelom 42–40 × 8

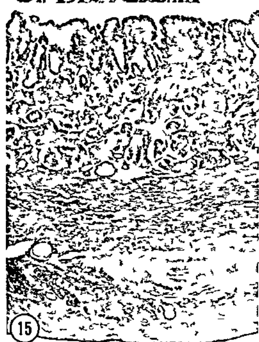
17 Another section of the gestation sac shown in figure 16 showing the allantoic stalk (arrow) 42–40 × 8

18 Another section of the gestation sac shown in figure 16 showing the antimesometrial hematome just starting to bulge toward the allantoic vesicle It is on the major endometrial lobe 42–40 × 8

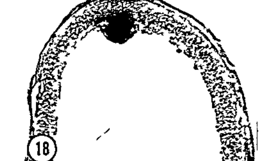

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## PLATE 2

### EXPLANATION OF FIGURES

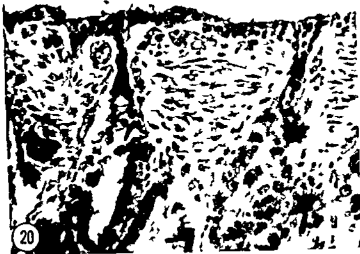
19 Detail of a portion of the mesometrial region of figure 16 showing the chorio vitelline placental labyrinth (above) the spongy zone and the unmodified glands of the deep glandular zone The spongy zone glands are hypertrophied greatly dilated and their walls folded Note the splanchnopleuric yolk sac (arrow) and just outside it the smooth chorion spanning an endometrial groove where the spongy zone is lacking The uterine lumen undercuts the margin of the chorio vitelline placenta 62–40 × 32

20 Detail of beginning chorio-vitelline placenta (earlier than fig 19) showing relatively straight and solid trophoblastic villi penetrating the gland lumina 62–168 × 200

21 Detail of figure 19 showing the chorio-vitelline labyrinth which is endotheliochorial in type The villi have vascular mesodermal cores (arrow) and are branched and tortuous Note the dark masses of gland symplasma at the tips of the villi 62–10 × 100

22 Detail of the chorio-allantoic labyrinth in the antimesometrial region shown in figure 16 Note the similarity to the chorio-vitelline placenta shown in figure 21 both are endotheliochorial 62–40 × 100


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## PLATE 2

### EXPLANATION OF FIGURES

19 Detail of a portion of the mesometrial region of figure 16 showing the chorio vitelline placental labyrinth (above) the spongy zone and the unmodified glands of the deep glandular zone The spongy zone glands are hypertrophied greatly dilated and their walls folded Note the splanchnopleuric yolk sac (arrow) and just outside it the smooth chorion spanning an endometrial groove where the spongy zone is lacking The uterine lumen undercuts the margin of the chorio vitelline placenta 62–40 × 32

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## PLATE 3

### EXPLANATION OF FIGURES

23 An intermediate stage of the chorio-allantoic placenta showing the labyrinth the spongy zone glands and a few glands (arrow) of the deep *zone Note the maternal* vessel in a septum 62–53 × 28

24 Detail of placenta shown in figure 23 showing the endotheliochorial labyrinth The maternal capillary endothelium is noticeably thick 62–53 × 100

25 *Margin of the antimesometrial portion of the zonary band of an intermediate* stage placenta showing the deep undercut (arrow) and the slanting direction *of the dilated glands 62–136 × 32*

26 Detail of labyrinth of definitive stage of the chorio-allantoic placenta showing the well developed villi and the sharply delineated zones of intimacy" between them 62–29 × 20

27 Intermediate placenta showing spongy zone glands diverging fanlike from a small area This borders a deep interlobular groove (arrow) *on the fetal surface* of the placenta 62–147 × 20

28 Definitive placenta showing the relatively thick labyrinth and the greatly compressed junctional and spongy zones Maternal vessels are prominent in the septa at the base and in the myometrium 62–47 × 11

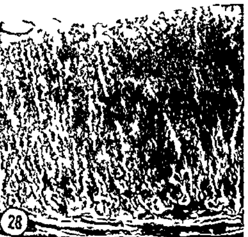

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## PLATE 4

### EXPLANATION OF FIGURES

29 Portion of antimesometrial region of specimen shown in figures 16–18 showing early hematomic pouches coalescing to form larger ones 62–40 × 32

30 More marginal portion of hematomic area shown in figure 29 showing separate small developing hematomic pouches with their closely apposed chorio allantoic vessels 62–40 × 32

31 Section of a definitive placenta through the hematome showing its folds or septa extending into its stalk and the dilatation with blood of the glands to which the stalk attaches ADFG 518 × 6 8

32 Section through part of the hematome and stalk of a late placenta showing the complicated folding of its lining and the connections of its cavity with those of the basal glands 62–135 × 6 5

33 Portion from figure 32 enlarged to show continuity of chorio allantois of labyrinth (upper margin) with that of hematome The tall epithelium and the brown pigment (black in illustration) of the highly folded absorptive lining of the hematome are obvious 62–135 × 16


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## PLATE 5

### EXPLANATION OF FIGURES

34 Detail of the base of the definitive placenta showing an interglandular *septum with its vessels complex folds and columnar epithelium* In the gland lumen at the right is a characteristic villous tip covered by columnar phagocytic epithelium 62–47 × 100

35 Detail at the base of the hematome stalk of a late placenta (same as fig 32) showing the fibrous zone surrounding the area where the stalk connects to the spongy gland zone Here the maternal capillaries and other small vessels are shrunken and their endothelium is degenerating They are strongly PAS positive and hence appear black in the photomicrograph Apparently maternal blood leaks from these vessels into the dilated glands and into the stalk of the hematome 62–135 × 32

36 Detail of a portion of the stalk of the late hematome in figure 32 showing a narrow channel between two folds of chorio allantoic membrane The tall columnar trophoblastic epithelium is packed with phagocytosed maternal erythrocytes Note the rich fetal capillary plexus at its base 62–135 × 200

37 Detail of a portion of the early hematome shown in figure 18 The trophoblastic epithelium is already tall columnar and actively phagocytic 62–40 × 200

38 Detail of a branched fold in a late hematome The tall phagocytic cells are full of maternal erythrocytes Although not shown here in most places the lumen would be completely occupied by mostly degenerating maternal erythrocytes and much pigment often in crystalline form The detached cells and degenerating cell fragments shown here are probably the result of postmorten changes before fixation of the tissue 62–37 × 200


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## PLATE 6

### EXPLANATION OF FIGURES

39 Detail of a portion of the junction of the hematome stalk and adjacent labyrinth of the late placenta shown in figure 32 The connective tissue strip to the right of center is the core of a chorio-allantoic villus Note that the trophoblast on its left side forms the epithelial lining of the stalk of the hematome while that on its right side forms the labyrinthine trophoblast enclosing the vessels lined by maternal endothelium 62–135 × 112

40 Detail of the margin of the placenta shown in figure 25 The central portion of the figure is occupied by a villus like fold of the chorio allantoic membrane just lateral to the endotheliochorial labyrinth At the right is the low columnar epithelium of the uterine wall and at the left a portion of the outer wall of one of the most lateral glands of the spongy zone The absence of uterine epithelium from this latter portion may be due to postmortem sloughing The trophoblastic epithelium of these marginal folds appears to be as actively phagocytic as it is at the tips of the villi throughout the placental base 62–136 × 200

41 Labyrinth of a late placenta 62–135 × 80

42 Detail of the labyrinth of the definitive stage of the placenta shown in figure 28 Note the thick maternal endothelium e lining the broad maternal tubules and the adjacent trophoblastic layer often deeply indented toward the tubules by fetal capillaries c 62–47 × 384


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# The Production of Congenital Malformations Using Tissue Antisera

## IV EVALUATION OF THE MECHANISM OF TERATOGENESIS BY VARYING THE ROUTE AND TIME OF ADMINISTRATION OF ANTI RAT KIDNEY ANTISERUM [1]

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ABSTRACT  Sheep anti rat kidney serum and rabbit anti rat kidney serum were utilized in a number of biological studies in order to clarify some of the qualitative and quantitative effects of this antiserum The teratogenic factor or factors present in these antisera can be characterized as follows The antiserum is (1) ineffective when administered by the oral route (2) the antiserum is teratogenic when administered by the intravenous intraperitoneal subcutaneous or intrauterine route (3) more effective by the intravenous and intraperitoneal route than by the subcutaneous route and (4) no more effective when injected into the uterine lumen of a pregnant rat than when given intravenously (5) uterine vascular clamping experiments and injection of the antiserum during the preimplantation period indicate that the teratogenic milieu persists for days following the administration of the antiserum (6) the antiserum can be considered a classical teratogenic agent because it produces a different spectrum of malformations on different days of gestation and concomitant fetal mortality and growth retardation although stage specificity is not as dramatic with the antiserum as with irradiation (7) although the exact mechanism of teratogenesis is not understood it was pointed out that fluorescent localization data and the experiments reported herein indicate a similarity between teratogenic antiserum and trypan blue (8) the importance of yolk sac pathology during the period of differentiation is discussed as it pertains to normal embryology teratogenesis and the utilization of animals in drug testing programs for the evaluation of teratogenic effects

The teratogenicity of rat kidney antiserum has previously been reported (Brent Averich and Drapiewski 61) and corroborated (David Mercier and Tuchman-Duplessis 63) Furthermore rat placental antiserum has also been demonstrated to be teratogenic (Brent 64a b 65 66a) Both these antisera produce a similar spectrum of congenital malformations in rat embryos exposed to the antisera during the early periods of differentiation (Brent 64a 65) As yet we cannot confidently state the exact series of events that result in congenitally malformed offspring In fact we are not certain of the primary site of action of the antiserum (embryo maternal organism placenta or extraembryonic membranes)

The purpose of this presentation is to further characterize the biologic aspects of antiserum induced teratogenesis by varying the route and time of injection of the antiserum It would be beneficial to know the similarities and differences between these antisera and other well known teratogenic agents Finally further characterization of the quantitative and qualitative properties of teratogenic serum may provide valuable direction to the necessary biochemical and immunologic studies

### MATERIALS AND METHODS

The method of obtaining timed pregnancies in our strain of Wistar rats has been described in previous publications (Brent 64a) The pregnant rat was anesthetized with sodium pentobarbital (30 mg/kg subcutaneously) on the day that the antiserum was injected If the maternal rat was pregnant for five or more days a laparotomy was performed and the number and condition of the embryonic sites

[1] Supported by N.I.H. grant HD 630 and 1 T1 HD 141

TABLE 1

*The teratogenic lethal and growth retarding effects of rabbit anti rat kidney serum when administered by the intravenous intraperitoneal subcutaneous or oral route to rats eight days pregnant*

| Route of administration | Dose in ml/100 g pregnant rat | Number of embryos (Pre-injection) | Per cent mortality | Term number | Fetuses weight ± S D | Per cent fetuses malformed |
|---|---|---|---|---|---|---|
| Intravenous | 0 125 | 58 | 10 5 | 52 | 4 51±0 37 | 42 3 |
| | 0 25 | 65 | 24 2 | 52 | 4 15±0 52 | 75 0 |
| | 0 50 | 80 | 48 9 | 41 | 4 19±0 67 | 95 2 |
| Intraperitoneal | 0 25 | 68 | 19 0 | 55 | 4 06±0 17 | 85 6 |
| | 0 50 | 87 | 49 4 | 44 | 4 04±0 21 | 79 6 |
| | 0 75 | 68 | 97 1 | 2 | 1 55 | 100 0 |
| | 1 0 | 71 | 100 0 | 0 | — | — |
| Subcutaneous | 0 25 | 79 | 5 1 | 75 | 5 21±0 502 | 5 3 |
| | — | — | — | — | — | — |
| | 0 75 | 68 | 20 5 | 53 | 4 12±0 60 | 81 2 |
| | 1 00 | 63 | 31 8 | 43 | 3 87±0 62 | 86 3 |
| Oral | 1 00 | 74 | 0 0 | 74 | 4 44±0 36 | 0 0 |
| | 2 00 | 59 | 5 1 | 56 | 4 93±0 41 | 1 9 |
| | 3 00 | 24 | 4 1 | 23 | 5 05±0 31 | 0 0 |
| Control | — | 97 | 8 2 | 89 | 4 80±0 31 | 1 7 |

TABLE 2

*The spectrum and incidence of malformations produced by intravenous intraperitoneal and subcutaneous administration of rabbi anti rat kidney antiserum to rat eight days pregnant*

| | Route of administration | | |
|---|---|---|---|
| | I V | I P | S C |
| Total live term fetuses from all dosage groups | 145 | 101 | 171 |
| Total live term fetuses with one or more malformations | 103 | 84 | 85 |
| Per cent live term fetuses malformed | 71 2 | 83 2 | 50 0 |
| | Per cent malformed fetuses with a particular malformation | | |
| Situs inversus totalis | 1 3 | 2 4 | 2 3 |
| Omphalocele and evisceration | 2 7 | 8 3 | — |
| Anencephaly | — | 2 4 | — |
| Meningocele | — | 1 2 | — |
| Encephalocele | 2 7 | 15 5 | 3 9 |
| Hydrocephaly | 59 7 | 75 0 | 67 4 |
| All eye malformations | 76 3 | 96 5 | 81 3 |
| Cleft palate | — | 4 7 | 4 6 |
| Facial clefts | — | 2 4 | — |
| Jaw and tongue malformation | 18 0 | 8 3 | 4 6 |
| Aortic arch malformation (total) | 6 9 | 25 0 | 9 8 |
| Diaphragm anomalies | 1 3 | 2 4 | 6 9 |
| Right renal agenesis | — | — | 2 3 |
| Left renal agenesis | 1 3 | 15 5 | 18 6 |
| Bilateral renal agenesis | 6 9 | 10 7 | 16 2 |
| Left ovarian agenesis | — | 1 2 | — |
| Left uterine agenesis | — | 2 3 | — |
| Bilateral testicular agenesis | 4 1 | — | 2 3 |
| Right sided cryptorchidism | — | — | — |
| Left sided cryptorchidism | 2 7 | 2 4 | 4 6 |
| Bilateral cryptorchidism | 6 9 | 13 1 | 6 9 |
| Bilateral absent or low set ears | — | 2 4 | — |
| Syndactyly | — | — | — |
| Limb hypoplasia | — | — | — |
| Recto vesical fistula | — | 1 2 | — |

were observed and recorded The incision was closed with silk sutures and metal wound clamps and the animal was injected or administered teratogenic kidney antiserum and allowed to go until the twenty first day of gestation at which time the maternal organism was sacrificed and the fetuses were delivered by cesarean section The fetuses were weighed fixed in Bouin s solution and dissected The mortality mean weight standard deviation of the fetal weights and the incidence of malformations were determined

There were three separate experiments dealing with modifications of route and time of administration of the teratogenic antiserum The first series of experiments consisted of the administration of teratogenic doses of antiserum to anesthetized eight day pregnant rats via the oral subcutaneous intraperitoneal or intravenous route (tables 1 2) The intravenous injection was administered via the tail vein The oral administration of the antisera was carried out with the aid of a small polyethylene catheter which was placed in the animal s stomach

In the second series of experiments the teratogenic antiserum was injected into the lumen of a pregnant rat uterus (fig 1 table 3) In order to make certain that the antiserum once placed in the uterine cavity did not leak out via the vagina or fallopian tube a ligature was placed at both the vaginal and ovarian ends of the uterus without interfering with the blood supply to that uterine horn (fig 1) Following the placement of the ligatures a small volume of highly concentrated antiserum was injected into the uterine lumen with a 30 gauge stainless steel needle The other uterine horn was similarly prepared and injected with saline rather than teratogenic serum (table 3)

TABLE 3

*Comparison of the effect of an intravenous injection of teratogenic sheep anti rat kidney serum and a comparable dose injected intraluminally into the isolated right uterine horn of an eight day pregnant rat*

| Agent injected | Route | Dose | Mortality | | Term fetal weight | | Malformation | |
|---|---|---|---|---|---|---|---|---|
| | | | Right horn | Left horn | Right horn | Left horn | Right horn | Left horn |
| | | | % | % | gm | gm | % | % |
| Saline | intrauterine | 0 5 ml | 14 2 | 0 | 4 239 | 4 240 | 0 | 0 |
| Sheep anti rat kidney serum | intrauterine | 140 mg (0 25 ml) | 100 | 100 | — | — | — | — |
| Sheep anti rat kidney serum | intrauterine | 70 mg (0 25 ml) | 100 | 100 | — | — | — | — |
| Sheep anti rat kidney serum | intrauterine | 35 mg (0 25 ml) | 95.2 | 52 9 | 2 998 | 3 212 | 100 | 100 |
| Sheep anti rat kidney serum | intravenous | 35 mg | 33 2 | 32 8 | 2 457 | 2 467 | 100 | 100 |
| Sheep anti rat kidney serum | intrauterine | 26 5 mg | 80 0 | 77 7 | 3 690 | 2 948 | 100 | 100 |
| Sheep anti rat kidney serum | intrauterine | 87 5 mg/kg approx 20 mg/rat | 34 7 | 14 2 | 4 128 | 4 252 | 86 6 | 75 |
| Sheep anti rat kidney serum | intravenous | 87 5 mg/kg approx 20 mg/rat | 11 7 | 16 7 | 4 006 | 4 099 | 53 5 | 60 |

[1] Ligature was placed at ovarian and cervical ends without interfering with blood supply to prevent injected material from leaking out via the fallopian tube and/or cervix

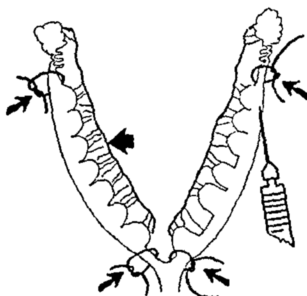


*Fig 1* The intrauterine injection of either saline or teratogenic serum was accomplished with a number 30 needle The cervical and ovarian ends of both uterine horns were ligated prior to the injection without interfering with the uterine blood supply The small arrows point to the ligatures and the large arrow points to the intact uterine vascular supply By concentrating the serum effective amounts could be administered in small volumes

The third series of experiments consisted of administering teratogenic antiserum by the intraperitoneal route on various days of gestation prior to the time of differentiation (table 4) It was previously reported that congenital malformations could be produced by injecting teratogenic antiserum on the seventh eighth ninth or tenth day of rat gestation (Brent, 64a) In these studies teratogenic kidney antiserum was administered after zero three and five days of rat gestation

The fourth series of experiments consisted of combining the uterine vascular clamping procedure with the intravenous injection of teratogenic rabbit anti rat kidney antiserum In previous reports we demonstrated that 30 minutes of uterine vascular clamping on the eighth day of gestation did not appreciably interfere with embryonic growth or produce excessive fetal mortality or malformations (Brent and Franklin 60, Franklin and Brent '64) Therefore, following the application of clamps to both the cervical and ovarian ends of one uterine horn an intravenous injection of teratogenic antiserum was administered to a pregnant rat on the eighth day of gestation Thirty minutes later the clamp was removed and the pregnant rat was allowed to go to term There were 54 pregnant rats who underwent the combined clamping procedure and injection of the teratogenic antiserum (table 5)

## RESULTS

The teratogenic lethal and gross retarding effect of rabbit anti rat kidney antisera administered by various routes is summarized in table 1 The operative control group had a malformation rate of 1 7% and a resorption rate of 8 2% The administration of massive doses of teratogenic serum via the oral route did not interfere with the embryonic development of the rat The intravenous intraperitoneal and subcutaneous route of administration all had some deleterious effect upon the embryo The subcutaneous route was the least effective of the three for

TABLE 4

*The teratogenic lethal and growth retarding effectiveness of a single administration of rabbit anti rat kidney serum on various days of gestation in the rat*

| Day | Dose of antiserum ml/100 g of rat | Number of embryos after 8 days of pregnancy | Number of term fetuses | Mortality | Term fetal weight | Malformations |
|---|---|---|---|---|---|---|
| | | | | % | g | % |
| 0 | 1 0 | 31 | 29 | 6 4 | 4 660 ± 0 37[1] | 6 8 |
| 3 | 0 5 | 51 | 48 | 5 8 | 4 762 ± 0 41 | 16 6 |
| 5 | 0 5 | 36 | 34 | 5 5 | 4 285 ± 0 52 | 47 0 |
| 7 | 0 5 | 66 | 36 | 45 4 | 3 990 ± 0 61 | 38 9 |
| 8 | 0 5 | 87 | 44 | 49 4 | 4 191 ± 0 44 | 95 6 |
| 9 | 0 5 | 87 | 54 | 38 0 | 4 110 ± 0 43 | 55 6 |
| 10 | 0 5 | 80 | 69 | 13 6 | 3 801 ± 0 57 | 42 0 |

[1] S D

TABLE 5

*The effect of combining the technique of uterine vascular clamping and the intravenous injection of teratogenic antiserum into eight-day pregnant rats*

| | Length of clamping | Dose of antiserum | Number of embryos | Mortality | Term f tal weight | Mal formations |
|---|---|---|---|---|---|---|
| | *minutes* | *ml* | | % | | % |
| Clamping alone | 30 | 0 | 74 | 6 7 | 4 59 | 3 9 |
| Clamping plus antiserum | 30 | 0.25 | 191 | 29 8 | 4 475 | 16 4 |
| Antiserum alone | 0 | 0.25 | 234 | 27.8 | 4 507 | 18 8 |

the embryos could receive over twice the amount of the antisera that was equivalent to the intravenous or intraperitoneal LD/50 dose and yet have only a 30% mortality In spite of this there was a dose of antiserum that could be given by any of these three routes that would result in malformations lethality and growth retardation Although the intravenous and intraperitoneal routes were fairly comparable at the high dose levels it was possible to produce malformations with significantly smaller intravenous doses than were listed in table 1 while these same small doses given by the intraperitoneal route were not teratogenic

The spectrum and incidence of malformations following the injection of teratogenic serum by various routes are summarized in table 2 The incidence and type of malformations are not too different following the administration of teratogenic antiserum by the three parenteral routes Those organ systems which are heavily involved following the administration of the antiserum by one route are similarly involved when the antiserum is administered by the other routes There was a lower incidence of malformations in the group administered the teratogenic serum subcutaneously probably because all groups of animals in this category received less than the embryonic LD/50 dose (table 1)

A comparison of the effectiveness of the intrauterine and intravenous route of administration yielded some very interesting results (table 3) When saline was injected into the ligatured uterine horn it was not surprising to find a slightly increased mortality in the embryos in the injected uterine horn although the incidence of malformations was no greater than in the control group If the intrauterine injection of concentrated teratogenic serum contained more than 70 mg of lyophilized teratogenic serum there was a 100% mortality in both uterine horns In the experimental groups in which some embryos survived it was apparent that the intrauterine method of administering the antiserum was no more effective than the intravenous route Furthermore the fetuses from the intrauterine group that were in the uninjected uterine horn were just as severely affected as were those fetuses in the horn that received the intraluminal injection of antisera (table 3)

Thus the embryos in the uninjected or control uterine horn had the same incidence of malformations resorptions and growth retardation as the embryos in the tied and injected uterine horn Furthermore the mortality and incidence of malformations are quantitatively the same following either the intraluminal or intravenous injection of the same dose of antiserum

The administration of teratogenic antiserum prior to and during the period of implantation yielded the most unpredictable results since malformations were observed in term fetuses whose mothers were injected with antiserum on the third and fifth day after conception (table 4) It is even more interesting to note that in the five-day group the term mortality was only 5 5% although the incidence of malformations was 47% Furthermore there was no evidence of increased mortality or growth retardation in the three-day group and yet there was a significant increase in the incidence of malformations Thus it is apparent that the injection of teratogenic antiserum from the third day post conception to at least the tenth day post

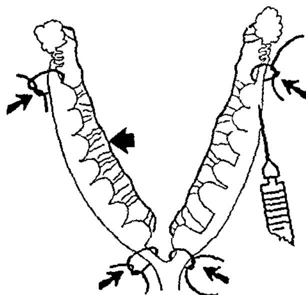


Fig 1 The intrauterine injection of either saline or teratogenic serum was accomplished with a number 30 needle The cervical and ovarian ends of both uterine horns were ligated prior to the injection without interfering with the uterine blood supply The small arrows point to the ligatures and the large arrow points to the intact uterine vascular supply By concentrating the serum effective amounts could be administered in small volumes

The third series of experiments consisted of administering teratogenic antiserum by the intraperitoneal route on various days of gestation prior to the time of differentiation (table 4) It was previously reported that congenital malformations could be produced by injecting teratogenic antiserum on the seventh eighth ninth or tenth day of rat gestation (Brent 64a) In these studies teratogenic kidney antiserum was administered after zero three and five days of rat gestation

The fourth series of experiments consisted of combining the uterine vascular clamping procedure with the intravenous injection of teratogenic rabbit anti rat kidney antiserum In previous reports we demonstrated that 30 minutes of uterine vascular clamping on the eighth day of gestation did not appreciably interfere with embryonic growth or produce excessive fetal mortality or malformations (Brent and Franklin 60 Franklin and Brent '64) Therefore, following the application of clamps to both the cervical and ovarian ends of one uterine horn an intravenous injection of teratogenic antiserum was administered to a pregnant rat on the eighth day of gestation Thirty minutes later the clamp was removed and the pregnant rat was allowed to go to term There were 54 pregnant rats who underwent the combined clamping procedure and injection of the teratogenic antiserum (table 5)

## RESULTS

The teratogenic lethal and gross retarding effect of rabbit anti rat kidney antisera administered by various routes is summarized in table 1 The operative control group had a malformation rate of 1 7% and a resorption rate of 8 2% The administration of massive doses of teratogenic serum via the oral route did not interfere with the embryonic development of the rat The intravenous intraperitoneal and subcutaneous route of administration all had some deleterious effect upon the embryo The subcutaneous route was the least effective of the three for

TABLE 4

*The teratogenic, lethal and growth retarding effectiveness of a single administration of rabbit anti rat kidney serum on various days of gestation in the rat*

| Day | Dose of antiserum ml/100 g of rat | Number of embryos after 8 days of pregnancy | Number of term fetuses | Mortality | Term fetal weight | Malformations |
|---|---|---|---|---|---|---|
| | | | | % | g | % |
| 0 | 1 0 | 31 | 29 | 6 4 | 4 660 ± 0 37 [1] | 6 8 |
| 3 | 0 5 | 51 | 48 | 5 8 | 4 762 ± 0 41 | 16 6 |
| 5 | 0 5 | 36 | 34 | 5 5 | 4 285 ± 0 52 | 47 0 |
| 7 | 0 5 | 66 | 36 | 45 4 | 3.990 ± 0 61 | 38 9 |
| 8 | 0 5 | 87 | 44 | 49 4 | 4 191 ± 0 44 | 95 6 |
| 9 | 0 5 | 87 | 54 | 38 0 | 4 110 ± 0 43 | 55 6 |
| 10 | 0 5 | 80 | 69 | 13 6 | 3 801 ± 0 57 | 42 0 |

[1] S D

fetal death growth retardation and mal formations Early injection of the anti serum practically eliminates the lethal effect while fetal growth retardation and malformation appear to be modified to a similar extent (table 4) Does this mean that the growth retarding and malforming factor or factors in the antiserum are the same or more closely allied than the factor which produces lethality? Until the pathologic immunoglobulins can be isolated in pure form one cannot determine whether all three pathologic effects are properties of one or more molecules

If one believes that the teratogenic milieu persists for several days then the vascular isolation of one uterine horn for 30 minutes following the administration of antiserum should not protect against malformations Thus failure to protect with the technique of uterine vascular clamping and malformation production follow in the injection of teratogenic serum during the pre implantation period are results that are in agreement

The purpose of injecting teratogenic serum directly into one pregnant uterine horn was to determine whether pathologic effects could be limited to the injected horn If this occurred then the mechanism of action of the antiserum would be partially clarified since it would eliminate the possibility that teratogenesis was the indirect effect of induced maternal metabolic disease and the result of a direct effect upon either the trophoblast uterus extra embryonic membranes or embryo The fact that the embryos in both uterine horns were equally affected indicates that the induction of maternal metabolic or physiologic disease cannot be eliminated by this experiment One explanation for this result is that the antiserum could not diffuse from the uterine lumen to the implantation site and therefore the antiserum was absorbed into the circulation just as if it had been injected intravenously or intraperitoneally thus affecting both uterine horns to the same extent

3 *Do these biologic experiments give any indication that the antiserum is teratogenic because it contains teratogenic immunoproteins?* Although we have evidence that the teratogenic factor is present in the gamma globulin fraction of the serum is non dialyzable and is inactivated by antiserum made against the gamma globulin from the teratogenic serum (Brent 66d) there is only minimal evidence from these biologic experiments to support the data obtained from immunochemical studies Although the ineffectiveness of oral administration of teratogenic antiserum is presumptive evidence that the teratogenic factor is a protein which is destroyed in the gastrointestinal tract it also could be a small molecule that is labile or not able to be absorbed from the intestine Thus although the immunologic data strongly supports the existence of a teratogenic immunoglobulin these biologic experiments were only of minimal help

4 *Are there important similarities and differences between teratogenic antiserum and other classical teratogenic agents?* Teratogenic antiserum can be considered a classical teratogenic agent which produces a wide spectrum of malformations during the period of rapid differentiation It is unusual in one respect in that it is harvested from mammals following the injection of mammalian tissue The type of malformations vary somewhat with the day of administration although the variation is not as great as following irradiation Growth retardation and embryonic mortality are intimately related to the teratogenic process The fact that injections as early as the third post-conception day in the rat result in malformations and that the immunoprotein localizes in the yolk sac membrane (Slotnick and Brent 66) are properties that characterize trypan blue which is a chemical teratogenic agent (Wislocki '20 Fox 58 Wilson Shepard and Gennar 63 Beck Spencer and Baxter 60) This is an instance where the biologic experiment and immunologic experiment reinforce an interesting similarity between a teratogenic agent prepared immunologically in a mammal and a chemical dye prepared in the laboratory If events prove that both these agents produce teratogenesis via yolk sac pathology we may have to reconsider the relative importance of this mechanism of teratogenesis If yolk sac pathology proves to be an important factor in teratogenesis then drug testing programs

conception results in an increased incidence of malformations in the term fetuses Although the incidence of malformations in the zero day group is three times greater than the control group there were only three malformed embryos in a term group of 29 embryos This incidence is not significantly different from the incidence in the control embryos at the 5% level of significance

The combination of uterine vascular clamping and the injection of teratogenic antiserum is summarized in table 5 It is apparent that the isolation of one uterine horn from circulating teratogenic serum for 30 minutes does not protect the isolated embryos from the effects of the circulating antiserum since the growth retarding lethal and malforming effects were similar in the clamped and non clamped embryos

## DISCUSSION

Biochemical and immunological techniques will undoubtedly encompass the final steps in clarifying the mechanism of action of teratogenic antisera We have reported that fluorescent labeled teratogenic sheep anti rat kidney gamma globulin localizes in the glomerular basement membrane, adrenal and to a lesser extent in the spleen and liver of the maternal rat (Slotnick and Brent 66) Furthermore this same labeled antiserum localizes very heavily in the developing yolk sac basement membrane during the period of early embryonic differentiation but does not localize in the tissues of the very young embryos in vivo Thus the fluorescent localization data support the latter two of the three possible mechanisms of teratogenesis (1) direct effect upon the embryos (2) direct effect on the trophoblast or extra embryonic membranes or (3) indirect effect on the embryos via induced maternal metabolic disease

The biological experiments reported in this manuscript can yield some information which is difficult to obtain from biochemical and immunologic experiments

1 *Does the route and rate of administration of the antiserum influence the quantitative and qualitative effects of teratogenic serum?* The experiments reported here indicate that the route of administration can modify the pathologic effects of the antiserum While oral administration was shown to be ineffective teratogenesis resulted when the antiserum was administered by any parenteral route When low doses of antiserum were administered parentally the intravenous route was the most effective and the subcutaneous route least effective At higher doses the intravenous and intraperitoneal routes were comparable and the subcutaneous route was still the least effective These data indicate that local inactivation or local adsorption can modify the effectiveness of injected antiserum These same phenomena could also be explained on the basis of variation in dose rate since it is likely that the subcutaneous administration of antiserum results in a lower peak serum level which is attained at a later time after administration With the subcutaneous injection or low doses administered by other routes there was unequal attenuation of the rate of fetal death growth retardation and malformations The significance of this will be discussed later

2 *How long after injecting the antiserum does the teratogenic milieu persist?* Malformations were produced by the administration of teratogenic antiserum as early as three days post conception Since the types of malformations produced by injections on three and five days post conception were qualitatively similar to those produced in seven and eight-day old embryos it seems likely that the teratogenic effect is functioning during the period of differentiation (8½ to 12 days) and therefore the teratogenic milieu can last for four to six days following injection of the antiserum in the rat This is further substantiated by the fact that although the malformation types are the same, the incidence progressively declines as one administers the antiserum further from the period of differentiation and closer to the time of conception The same dose of antiserum produces a malformation rate of 16 6% 47 0% and 95% when injected on three five and eight days post conception respectively Furthermore attenuation of pathologic effects of the antiserum by earlier injection causes a disproportionate change in the incidence of

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will have to consider this possibility when choosing appropriate experimental animals (Brent, 64c) This would indicate that more must be learned about yolk sac function in mammals during the period of differentiation in order to add to our understanding of the variable importance of yolk sac function in various mammalian species

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